*Author:*
**Gracida Juarez, Carlos A**

*Title:*
**Competitive Behaviour, Impact and Success of Invasive Tilapia (Oreochromis spp.) in Quintana Roo, Mexico**

# Competitive behaviour, impact and success of invasive tilapia (*Oreochromis* spp.) in Quintana Roo, Mexico

Carlos Alberto Gracida-Juarez

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Representation of the Nile tilapia from Egyptian culture, circa 1556 – 1060 before our era.

Musee de Louvre.

**Abstract**

Tilapia cichlid fishes of the genus *Oreochromis* are invasive across the world's tropical freshwaters. In Quintana Roo, Mexico, tilapia are now widespread, but their impacts on indigenous freshwater fauna are unclear. This thesis reports investigations of effects of *Oreochromis* tilapia on native fish assemblages in Quintana Roo, following initial reports of their establishment in the 1980s and 1990s. Competitive interactions between invasive Nile tilapia (*Oreochromis niloticus*) and native Mayan cichlid (*Mayaheros urophthalmus*) were evaluated. The Nile tilapia was more active and aggressive than the Mayan cichlid, and better able to withstand the elevated temperatures and lower oxygen concentrations that negatively affected the native species. These results suggest Nile tilapia has broad tolerance of extreme environmental conditions that could favour invasive success of this species. Environmental DNA (eDNA) metabarcoding was evaluated as a tool for surveying fish communities invaded by tilapia. Focussing on Lake Caobas, a positive association was found between the number of eDNA reads of a species, and the number of captures of individual species using conventional net sampling. Moreover, using eDNA it was able to sample 18 of the 20 known species known from the lake, including invasive *Oreochromis*, while conventional netting sampled only 14 species. These findings support use of metabarcoding in monitoring. To explore the impact of *Oreochromis* on native fish assemblages, six lakes were surveyed using conventional net sampling. Tilapia were very rare, and only captured in three sampled lakes. Moreover, tilapia presence did not appear to influence native fish assemblages, and instead dissolved oxygen was the strongest predictor of species diversity. In these systems, native predators may regulate tilapia abundance and promote resistance to invasion. In conclusion, it is recommended that monitoring of both invasive feral tilapia and the native fauna, while providing informed guidance during the development of aquaculture, would lower the risk to native fish assemblages of the region.

**Acknowledgements and dedication**

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**Authors declaration**

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed Date: 7 April 2020

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# Chapter 1

# General Introduction

### 1.1 Biological invaders

A biological invader is a species of plant, animal or micro-organism which colonizes and spreads into new biogeographic areas beyond their native range. The introduction of invasive species is typically associated with human activity, either due to intentional or inadvertent transport (Di Castri 1990). The establishment and spread of invasive species has occurred at an accelerated rate over recent decades, leading to negative impacts on native biodiversity and ecosystem function (Chandra & Gerhardt 2008). Biological invasion is now an issue of global concern, and invasive species have been listed as second in importance as a cause of biodiversity loss, after habitat loss (Walker & Steffen 1997). Their annual economic impact has been stated to be as high as $1.4 trillion worldwide, which is nearly 5% of the global economy (Chandra & Gerhardt 2008). Across only six countries, the United States, United Kingdom, Australia, South Africa, India and Brazil, the collective annual economic impact of invasive species has been estimated to be US$ 314 billion, with impacts mainly affecting agriculture and the wider environment (Pimentel *et al.* 2001). Examples of invasive species include the weeds *Lantana camara* and *Opuntia ficus-indica* that affect the agriculture production in India and South Africa; and the invasive sparrow (*Passer domesticus*) and starling (*Sturnus vulgaris*) that cause an annual US$1 billion in loss in the USA. Further examples include the European rabbit (*Oryctolagus cuniculus*) that impacts the agricultural economy in the UK and Australia, and numerous arthropod and plant pathogens that cost billions to the agriculture sectors of India, the USA, South Africa and Brazil (Pimentel *et al.* 2000; Pimentel *et al.* 2001).

**1.2 Impacts of invasive species**

Invasive species are typically considered to be capable of successfully competing for space and food against native species (Lowe *et al.* 2000), and can often drive environmental changes within the invaded ecosystems making them less suitable for the original native community (Didham *et al.* 2005). Invasive species can also modify the interactions between native species within invaded ecosystems (Gallardo *et al.* 2016), and they can also spread infectious diseases contributing to declines in native populations (Crowl *et al.* 2008). The negative impacts on the local and national economies throughout the world can be substantial. For example, in the United States the ~50,000 established non-indigenous species cause estimated economic losses of $125 billion per year (Pimentel *et al.* 2000).

There are many factors associated with success of invasive species. In freshwaters, the spread of invasive species is limited by natural environmental filters, including factors such as upper and lower temperatures, oxygen and salinity, and food availability. There are concerns that global warming will promote further spread of freshwater invaders and their negative impacts will increase (Rahel & Olden 2008). Specifically, climatic warming is predicted to increase the accessibility of temperate and subtropical freshwater habitats to warmer water invaders, therefore expanding the number of native species exposed (Hellman *et al.* 2008). Collectively, invasive species may represent one of the biggest challenges for conservation biologists, in addition to global warming, the wildlife trade, and overexploitation (Allendorf & Lundquist 2003).

Aquatic invasions can be particularly problematic. The red-eared slider (*Trachemys scripta elegans*), native to Southern United States and Northern Mexico, now established in all the continents except Antarctica following introductions due to the pet trade, has been recorded

outcompeting native terrapin species (Lambert *et al.* 2019). In South Africa, rainbow trout (*Oncorhynchus mykiss*), largemouth bass (*Micropterus salmoides*) and common carp (*Cyprinus carpio*), threaten 60% of native freshwater fish species, with 11 species already reported as extinct. Other invasive species in South Africa include the mosquitofish (*Gambusia affinis*) and Mozambique tilapia (*Oreochromis mossambicus*); these are now well established persistent species and potentially problematic (Pimentel et al. 2001, Olds *et al.* 2011). In the USA, at least 138 invasive fish species are established in the country, causing damage to the sports fishing industry, and at least 44 native species are now considered at risk as a direct consequence of the invasions (Pimentel *et al.* 2001).

The North American Great Lakes collectively form one of the largest expanses of freshwater in the world. The region is also one of the most invaded with an estimated 184 alien species. One of the most notable invaders is the zebra mussel (*Dreissena polymorpha*), a species that causes significant impacts to biodiversity by outcompeting and smothering native species. This species also affects the hydropower industry and drinking water treatment plants by clogging the water intake pipes of industrial facilities. The economic impact from control measures is estimated to be US$ 800 million annually (Escobar *et al*. 2018).

Similarly, the invasive Asian clam (*Corbicula fluminea*), also outcompetes native species and damages infrastructure in the North American Great Lakes region. This species can survive when are consumed at temperatures below 21°C and pass through the gut of the blue migratory catfish (*Ictalurus furcatus*), contributing to the dispersion of these invasive bivalves (Gatlin *et al.* 2013). It has also been shown to be possible for invasive aquatic species to faciliate the establishment of other invasive species in this heavily invaded region. For example, in experimental mesocosms, the presence of the zebra mussel enhances the

dominance of two closely-related macrophytes, *Elodea canadensis* and *Elodea nutalli*. This feature may increase the impact of the zebra mussel when they co-occur with these invasive plants in the wild (Crane *et al.* 2020).

Invasive arthropod species are also of particular concern, particularly freshwater crayfish. These arthropods are primarily distributed as a food source, but are also spread through the aquarium pet trade, used in schistosomiasis vector snails control, and employed as live bait (Gherardi, 2007). When populations establish, these invasive crayfish species integrate easily into the food web at many levels. Since they have capability to travel long distances overland, they are also able to readily invade new aquatic systems (Ackefors, 1999; Battisti & Scalici, 2020; Krieg & Zenker, 2020).

Key crayfish species causing concern include signal crayfish (*Pacifastacus leviusculus*) and spiny-cheek crayfish (*Orconectes limosus*) in Europe and Japan, Danube crayfish (*Astacus leptodactylus*) in Europe, and the common yabby (*Cherax distructor*) in Africa. Other recently introduced species may lead to problems shortly, as is the case for the red-clawed crayfish (*Cherax quadricarinatus*) in Ecuador (Gherardi, 2007). The impacts of invasive crayfish species go from subtle behavioural modifications of resident species, to altered energy and nutrient fluxes within invaded habitats. At the community level, their impact can be particularly strong when native species that lack defence adaptations become prey for invasive crayfish (Nystrom *et al.,* 2001).

The negative effects of invasive crayfish on native crayfish become compounded when they build higher densities than the native species. At this point they can overharvest the existent macrophytes, which can lead to increase the production of algae, causing turbidity,

decreasing light penetration and consequently the regeneration of macrophytes and periphyton. Also, invasive crayfish may change the macroinvertebrate composition in lentic systems, due to their specific consumption habits, increasing the abundance of those species less vulnerable to the crayfish presence (Gherardi, 2007).

**1.3 Effects of invasive fish species - hybridization**

Hybridization commonly occurs in nature and is widely observed in fishes. However, the introduction of non-native species can lead to unprecedented levels of hybridization and associated loss of genetic purity in the native species (Whitney & Gabler 2008). Moreover, such hybridization between invasive and native species is becoming increasingly widespread due to human-mediated movement of the species (Allendorf *et al.* 2015). For example, in African freshwaters, hybridization between indigenous *Oreochromis* tilapia species and invasive Nile tilapia (*Oreochromis niloticus)* is a major cause of native species decline (Moralee *et al.* 2000; D'Amato *et al.* 2007). Specifically, hybridization has been reported between *O. niloticus* and several native species, including the Mozambique tilapia (*Oreochromis mossambicus)* in South Africa (Moralee *et al.* 2000; D'Amato *et al.* 2007) and the Singida tilapia (*Oreochromis esculentus*) in the Lake Victoria catchment (Angienda *et al.,* 2010). In this case the hybridization appears to be facilitated by the relevantly recent divergence of the *Oreochromis* species, which ensures they remain reproductively compatible (Costa-Pierce, 2003; D'Amato *et al.,* 2007). Importantly, it is also possible that hybridization between multiple invasive species can generate new successful phenotypes. In Lake Itasy, Madagascar, hybridization between the longfin tilapia (*Oreochomis macrochir*) and *O. niloticus* between 1963 and 1969 resulted in a 'hybrid boom'. Hybrids comprised up to 74% of fish catches, and

eventually individuals with the *O. macrochir* phenotype were lost from the population (Daget & Moreau, 1981; Moralee *et al.,* 2000).

The result of hybridization is often unpredictable. It can result in the decline or extinction of one or both parent populations, as reported for *O. mossambicus* and *O niloticus* in Sri Lankan reservoirs (Amarasinghe & De Silva, 1996), or facilitate successful invasion as seen in Lake Itasy. In most cases, however, the outcome is unclear as where multiple invasive congeneric species have been introduced, the true species composition of the invaders is unknown. For example, at least four *Oreochromis* tilapia species are established in the Americas, including *O. niloticus*, *O. mossambicus*, blue tilapia (*Oreochromis aureus*) and Wami tilapia (*Oreochromis urolepis*) (Cassemiro *et al.* 2018). Although several combinations of these species are known to hybridize successfully, there is very little information on the extent of hybridization that has taken place across their invaded range, and whether this has affected their impact on the natural environment.

**1.4 Effects of invasive fish species - competition**

Competition for food and space is common in nature, and these interactions are mediated by the availability of resources, and the biological traits of species. Where invasive and native species interact, environmental conditions can determine the relative success of the species. For example, in northern Italy the invasive mosquitofish (*Gambusia affinis)* is found alongside the killifish (*Aphanius fasciatus),* and studies of abundance in the field have shown that higher salinity levels appear to favour the native killifish over the invasive mosquitofish. Laboratory trials showed that salinity can mediate competition interactions between the species. Specifically, as salinity increased, aggressive behaviour directed towards the native killifish by

the invasive mosquitofish declined. The decrease in aggression then enabled higher food intake by the native killifish (Alcaraz *et al.* 2008).

The density of the invasive species plays an essential role in their success. It is typically the case that as densities of invasive species increase, so do any negative impacts on native species. However, in some cases high densities of invaders can lead to a reduction in the negative effects on native species, as intraspecific interactions increase. One example of this comes from the round goby (*Neogobius melanostomus*), a species original from the Caspian Sea which has invaded the North American Great Lakes region. In experimental mesocosms, the presence of low densities of invasive round gobies led to significant reductions in the growth of native white suckers (*Catostomus commersonii*) and Johnny darters (*Etheostoma nigrum*), but at high densities of round gobies the growth of the native species mirrored that of the no round goby controls. This result was presumed to be due to a high intensity of intraspecific interactions between round gobies at the highest densities, that diminished their ability to compete effectively with the native species (Kornis *et al.* 2014).

Temperature can take an important role in determining the abundance of limited resources and the relative performance of native and invasive species. In the Rocky Mountains of North America, three species of river fish are present and differ in their thermal tolerances which, in turn, influences their distribution along the altitude gradient (Rahel & Hubert; 1991). Specifically, the higher altitude cooler waters are occupied by the native brook trout (*Salvelinus fontinalis),* the medium altitude waters are occupied by the invasive brown trout (*Salmo trutta*), while the lower altitude warmer waters are occupied by the creek chub (*Semotilus atromaculatus*). Laboratory experiments of competitive ability across the temperature range demonstrated that the distributions are paralleled by competitive

performance across the temperature gradient. Most notably, creek chub were capable of outcompeting the trout species at warmer temperatures of 24 and 26°C, as the elevated temperatures led to increased stress in trout (Taniguchi *et al.* 1998). Future temperature increases as a consequence of global warming are likely to modify these fish assemblages by mediating shifts in the relative performance during competitive interactions.

**101.5 Effects of invasive fish species - predation**

Predators can take one of two often mutually exclusive roles in aquatic systems (Thorp 1986). Some predators may significantly influence community diversity by reducing population sizes below carrying capacity and limiting competitive exclusion. By contrast, other predator species take only a minor role in the ecology of modern communities, despite taking an important historical role in determining the composition of the current species pool. In such cases, the influential role of a predator on the structure of communities may only become evident when the predator is introduced into non-native habitats, where ecologically naïve species are present that have not co-evolved (or at least co-occurred) with that predator over evo-ecological timescales. In either of these cases, invasive predators can have significant impacts on native fish communities in the invaded range.

Largemouth bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis macrochirus*) are predatory fish species originating in North America, and they have invaded more than 70% of Japan's freshwaters. The presence of these species in Japanese farm ponds has been correlated with low diversity of native species (Yonekura *et al.* 2004). Experimental work comparing ponds with and without these predators demonstrates that together they can

suppress the abundance of multiple native species, including native topmouth gudgeon (*Pseudorasbora parva*), *Rhinogobius* gobies and the lake prawn (*Palaemon paucidens*), as well as exotic red swamp crayfish (*Procambarus clarkii*) (Maezono & Miyashita 2003). The predation not only leads to changes in diversity, but also fundamental properties of these pond ecosystems. Maezono *et al.* (2005) found that largemouth bass predation on the red swamp crayfish led to both an increase in macrophytes and eutrophication in experimental ponds.

The most spectacular example of the effects of introduction of native predators in freshwaters comes from introduction of Nile perch (*Lates niloticus*) into Lake Victoria in the 1950s, resulting in the direct loss of more than 200 species of native haplochromine cichlid fish that were naïve to this invasive species (Worthington & Lowe-McConnell 1994). Once the Nile perch established, there was substantive economic growth based on the industrialised fishery for the species. However, the traditional fisheries for native haplochromine cichlids collapsed, and demand for fuelwood used to preserve harvested Nile perch resulted in widespread deforestation around the lakeshore. Collectively, the extent of the ecological and socioeconomic impact of this introduction means that this is now widely considered to be one of the greatest environmental disasters to have faced freshwater ecosystems (Aloo *et al.* 2017).

### 1.6 Cichlid fishes in the "Tilapia" group

The cichlids are a species-rich family of teleost fishes with a broad natural distribution covering the Neotropics, Africa, Madagascar, the Middle East and the Indian subcontinent (Nelson 1994). Approximately 1,700 species are formally described, however many hundreds

of informally recognised taxa are known, particularly in the Great Lakes of East Africa, and the family may comprise as many as 3000-4000 species (Kullander 1998; Salzburger 2018). Due to the extent of the diversification of the cichlids in behaviour, morphology and ecology, they have been intensively studied as model system by evolutionary biologists over 50 years (Fryer & Iles 1972; Salzburger 2018).

Cichlid fishes from Africa and the Middle East comprise a monophyletic clade known as the "African radiation". Within this group there are multiple evolutionary lineages (tribes) that have historically been grouped under the general term "Tilapia" (Dunz & Schliewen 2013). This group of cichlids tend to have a superficially similar deep body shape, tend to occupy lakes or the slow-flowing stretches of rivers, and have a generalist diet comprising plankton, macrophytes, vegetative detritus and benthic invertebrates. One of the most diverse of the "Tilapia" tribes is the Oreochromini, that comprises ten genera, including *Oreochromis*. This is the most notable of the genera because of their relatively high species richness for the "Tilapia" genus (37 described species; Ford *et al.* 2019), but also their importance for capture fisheries and aquaculture across Africa. Moreover, several of the species have been used to develop capture fisheries and support aquaculture across other tropical regions of the world, including SE Asia, China and the Neotropics (McKaye *et al.* 1995; Kumar 2000; Canonico *et al.* 2005).

### 1.7 Importance of "Tilapia" in aquaculture

Cichlid fishes in the "Tilapia" group, hereafter referred to as "tilapia" have significant economic importance and are essential source of protein in human diets, particularly in Africa (Pullin & Lowe-McConnell 1982). Collectively, tilapia are known as the 'aquatic chickens',

because high protein yield can be achieved in a relatively short amount of time in aquaculture conditions, from subsistence units to intensive fish hatcheries. In some Asian countries and in Mexico, nearly all of the tilapia production is consumed domestically, contributing to food security for such societies (Canonico *et al.* 2005). Widespread exploitation and aquaculture of tilapias have led to their introduction in all tropical regions around the globe (Figure 1.1).

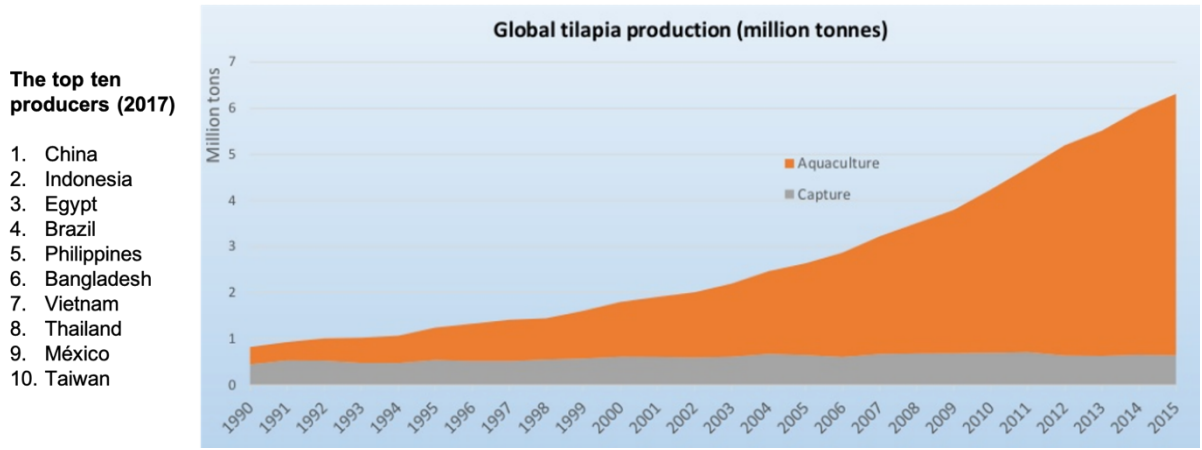



**Figure 1.1.** Global tilapia production from 1990 to 2015 (reprinted from Cai *et al.* 2019).

The most widely used of the tilapia species is the Nile tilapia, which is naturally distributed across West Africa, the Nile River basin, and as far south as Lake Kivu and Lake Tanganyika (Trewavas 1983) (Figure 1.2). The species is renowned for a broad environmental tolerance, particularly low oxygen, high temperature and highly eutrophic conditions (Canonico *et al.* 2005). It also has omnivorous feeding habits, rapid growth and can reach large body size of over 60 cm total length. It is a maternal mouthbrooder, with females capable of brooding hundreds of offspring in a single brood. These traits make it a useful target species for aquaculture and have also resulted in its widespread use in initiatives to improve capture

fisheries. It has also been introduced for mosquito control and sport fishing (Trewavas 1983; Zengeya 2011). However, the traits that make the species useful also predispose it to the successful establishment of feral populations, which are now present in at least 114 countries (Deines *et al.* 2016). These include tropical and sub-tropical countries across Asia, Africa and the Neotropics (Costa-Pierce 2003; Canonico *et al.* 2005). The species is now particularly widely distributed across Africa, and in countries such as Tanzania, most river systems are now colonised (Shechonge *et al.* 2019). In principle, isolated river basins that are still not colonised could act as ark sites enabling the conservation of indigenous congeneric species.

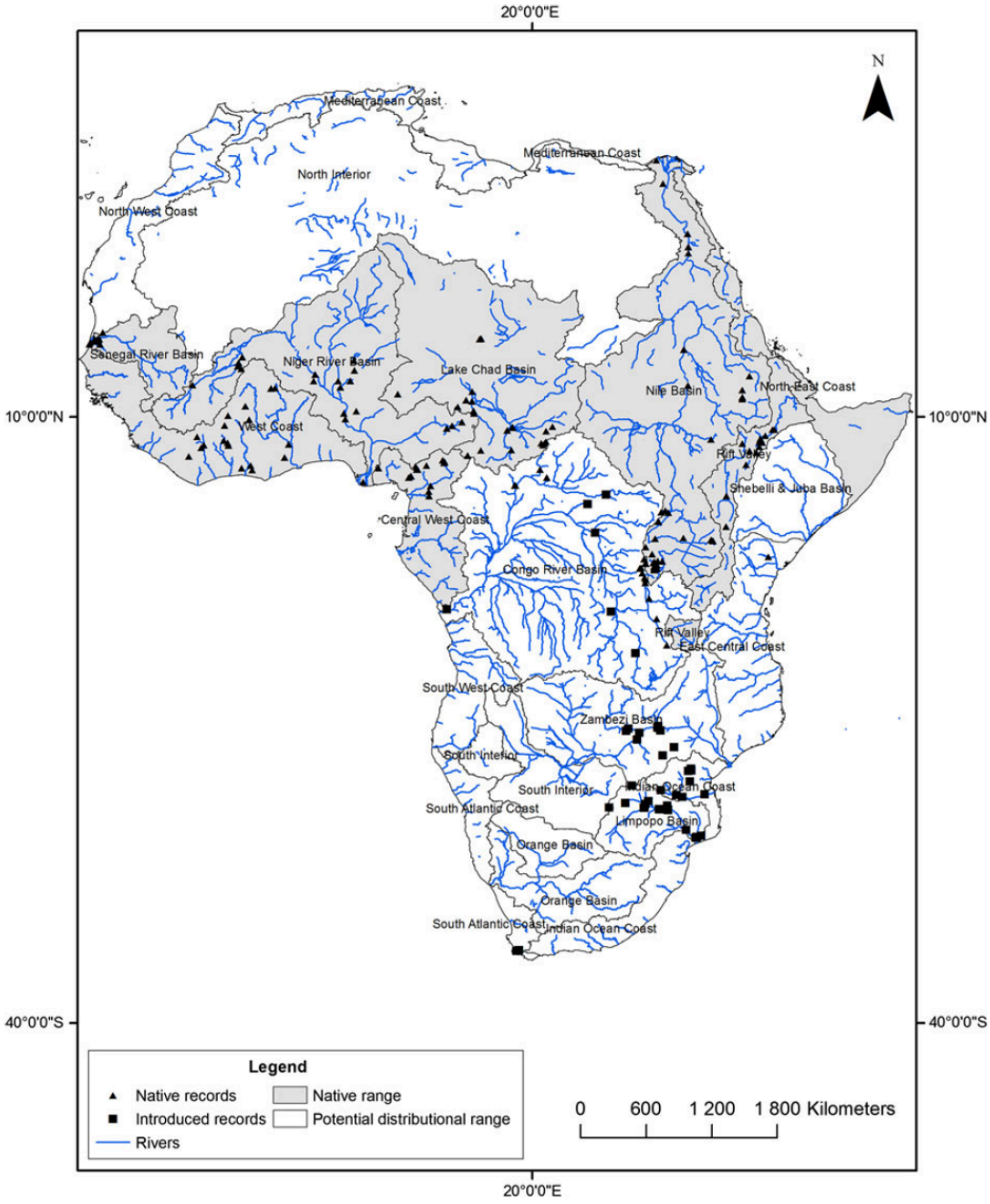



**Figure 1.2.** Records of Nile tilapia in Africa within its native range (▲, grey shaded area), and introduced records (■) (reprinted from Zengeya *et al.* 2013).

### 1.8 Impact of tilapia on native fish communities.

The promotion of tilapia as a viable source of fish protein in developing countries has contributed to its spread in many tropical countries and brought with it environmental challenges. Perhaps the main concern with the introduction of tilapia into novel aquatic

systems is their potential to compete successfully with native species (Canonico *et al.* 2005; Zengeya & Marshall 2007). Specifically, aggressive dominance may permit them to exclude indigenous species when competing for limited space or food resources (Holway and Suarez 1999).

There are now several experimental studies that suggest competition between native species and introduced tilapia may be taking place, to the detriment of native species. The spotted tilapia (*Pelmatolapia mariae*) has invaded freshwaters in southern Florida and co-occurs with native *Lepomis* sunfishes. In experimental trials, spotted tilapia exhibits higher levels of aggression and boldness than the sunfish, enabling the spotted tilapia to dominate competitive disputes and claim territory (Brookes and Jordan 2010). Nile tilapia is also known to be an aggressive competitor for resources (Freitas & Volpato 2008; Barreto *et al.* 2011). In China, manipulative experiments have shown that Nile tilapia had a negative influence on the growth of the native mud carp (*Cirrhinus molitorella*), mainly due to reductions in the availability of food for native species (Gu *et al.* 2014). In Brazil, Nile tilapia is sympatric with the native pearl cichlid (*Geophagus brasiliensis*) (Sanches *et al.* 2002), and in experimental conditions, Nile tilapia is considerably more aggressive and dominant than the pearl cichlid. Moreover, the differences in dominance persist even in cases were Nile tilapia are smaller than pearl cichlid (Sanches *et al.,* 2012). The loss of territorial space for native species may be particularly important in habitats where predators are abundant. Experimental work has shown that when *O. niloticus* displace the native sunfish (*Lepomis miniatus*) from shelter in the presence if piscivorous largemouth bass, the sunfish have the considerably lower rates of survivorship (Martin *et al.* 2010). Collectively these studies imply that native species can experience reductions in fitness where niche overlaps occur with invasive tilapia species.

Moreover, there is also evidence that this competition can lead to reduction in abundance of native species, as reported from fisheries in China (Gu *et al.* 2015).

In addition to the negative impacts caused by competition between native species and introduced tilapia, the presence of the invader can have indirect effects on native biota through other mechanisms. Invasive tilapia species can carry harmful parasites from their native range that are co-introduced to the non-native range, before later being transferred to indigenous species naïve to the parasite (Šimková *et al.* 2019). Exotic tilapia can also promote change at different trophic levels in communities. The presence of Nile tilapia, through can lead to an increase in the presence of nutrients that promotes change in the phytoplankton community. This can be achieved by the feeding behaviour that leads to a resuspension of nutrients in settled sediments, but also through the regular consumption and excretion of nutrients ensuring they are recycled in water column. In experimental enclosures in Furnas reservoir in Brazil, Nile tilapia increased the nitrogen and phosphorus by up to 260% and 540%, respectively. Resulting changes to the phytoplankton community and its composition, included increases in the mucilaginous chlorophytes. Since tilapia can contribute to the eutrophication of a water body, they must be used cautiously in aquaculture to avoid unexpected environmental degradation (Figueredo & Giani 2005).

**1.9 Importance of tilapia for aquaculture in Mexico**

Invasive fish species records in Mexico increased significantly between the 20th and 21st century. In 1904, the number of exotic species was four, and between 1949 and 1969 reports mentioned seven alien species. In 1983 there were fifty-five reported exotic species, and 94 in 1997 (Contreras-Balderas, 1999). By 2008, reports indicated 113 exotic fish were species distributed in the national territory (Contreras-Balderas *et al.,* 2008).

Tilapia are thought to have been first introduced into the Americas between the late 1960s and early 1970s (Pullin *et al.,* 1997). However, their large-scale production and international trade developed between the 1980s and 1990s. By 1998, total tilapia production in the Americas was 204,267 tonnes, of which 37% was *O. niloticus*, 20% was *O. aureus*, and the remaining 43% classified as "*Oreochromis* sp*."* (Watanabe *at al.* 2002). By the end of the 1990s, some 94,279 tonnes of tilapia were produced in Mexico, of which ~90,000 tonnes were consumed within the country (Fitzsimmons 2000). By 2000, it was reported that there were 1000 established tilapia farms producing regularly in Mexico. By 2010, Mexico continued to be the top tilapia producer on the continent, although there was strong growth in Cuba and Brazil (Fonticella & Sonesten 2000; Fitzsimmons 2000). In part, the continued growth is to meet the demand for tilapia in the USA, which grows at an average of 20% each year (Posadas 2000).

Tilapia now represent the third largest "seafood" product by weight in Mexico, after sardines (*Sardinops sagax caerulea*) in the Gulf of Baja California, and tuna (*Thunnus albacares*), and the fourth most valuable after shrimp (*Panaeus californiensis*, *Panaeus stylorostris*, *Panaeus vannamei*), tuna and octopus (*Octopus maya*, *Octopus vulgaris*). The Mexican states with the largest production are Michoacán, Veracruz, Tabasco and Chiapas, the last three located in Mexico's Neotropical region (Fitzsimmons 2000b). Several tilapia species were introduced to Mexico in the 1960s and 1970s. Mozambique tilapia (*O. mossambicus)* and blue tilapia (*O. aureus*) were first introduced in 1964, with Nile tilapia, Wami tilapia (*O. urolepis*) and red belly tilapia (*Coptodon zillii*) introduced in 1978 (Pullin *et al.* 1997). Mozambique tilapia was the species with the highest production in the initial years after tilapia introduction. By the late 1990s, Nile tilapia and blue tilapia replaced Mozambique tilapia production, with blue

tilapia cultivation particularly prevalent in the south of the country. Nowadays, every Mexican state is a tilapia producer, and Nile tilapia is the most cultivated species. Feral populations reported from many of the country's freshwater systems, with records of the Nile tilapia in the environmental being commonplace on the Global Biodiversity Information Facility biodiversity database (Figure 1.3).

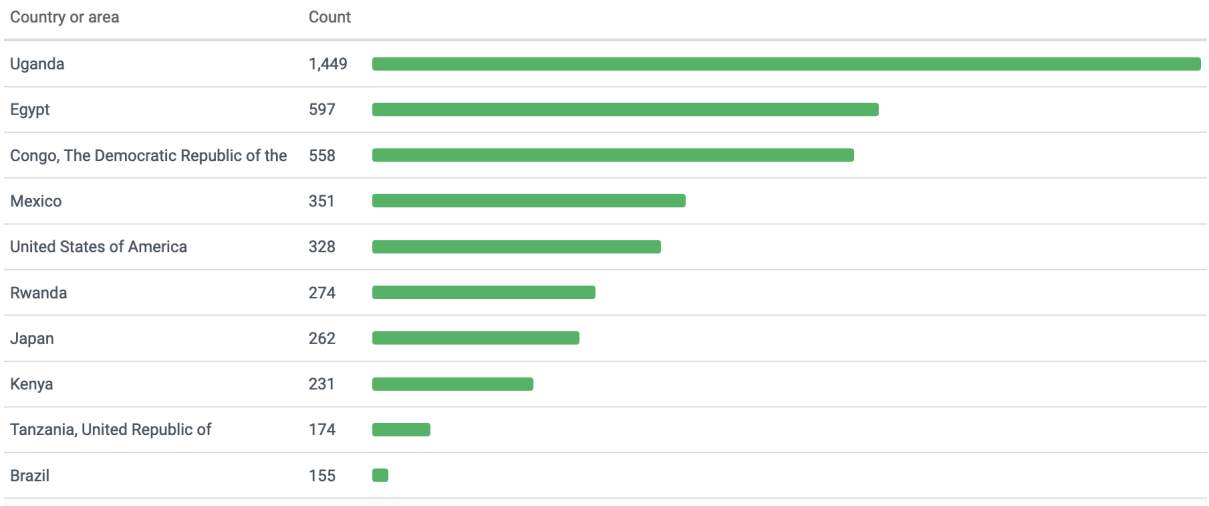



**Figure 1.3.** The ten countries with the higher number of occurrences of Nile tilapia within the Global Biodiversity Information Facility (GBIF) database. Note the species is native to Uganda, Egypt, Rwanda, the northern part of Kenya and the eastern part of the Democratic Republic of Congo, but invasive in all other countries. Data downloaded 16 March 2020. (https://www.gbif.org/species/4285694/metrics).

In Mexico, many of the occurrences of tilapia species in natural environments are a consequence of deliberate release. The distribution of tilapia into reservoirs behind dams has been a planned strategy to enable integrated fisheries and energy generation (Fitzsimmons 2000b). Fry-producing farms were built alongside the dams, and Nile tilapia now coexists with the native fish that inhabited the area before the creation of reservoirs. Tilapia cage

aquaculture is present in large water bodies, and reported escapes have been linked to activities of native crocodiles, and their malfunction due to flooding (Schmitter-Soto & Caro 1997; Fitzsimmons 2000b). Although a substantial amount of the production in the country is in small aquaculture ponds, these are generally considered less problematic for the spread of invasive tilapia species due to their isolation from natural water bodies.

Technological developments are changing the tilapia production mechanisms in Mexico. The most important states developing technology and increasing its production are Sonora and Chihuahua in the north of the country, where they have developed some mixed production models for shrimp and tilapia, and have experimented with production in marine water with positive results (Fitzsimmons 2000).

**1.10 Distribution and habitat of tilapia and native cichlid fishes in Mexico**

Tilapia are distributed mainly in the Neotropical region of Mexico, across both the Pacific and Atlantic slopes of the country (Figure 1.4). Estimation of the distribution of suitable habitat suggests that a high proportion of the national territory is suitable for the species to survive, if the species continues spreading (Figure 1.4). The distribution of this suitable habitat largely concurs with the Mexican states that most produce this fish, namely Michoacán, Veracruz, Tabasco and Chiapas. The Yucatan Peninsula, to the south-east of the country, is highlighted as representing suitable habitat and having records of species.

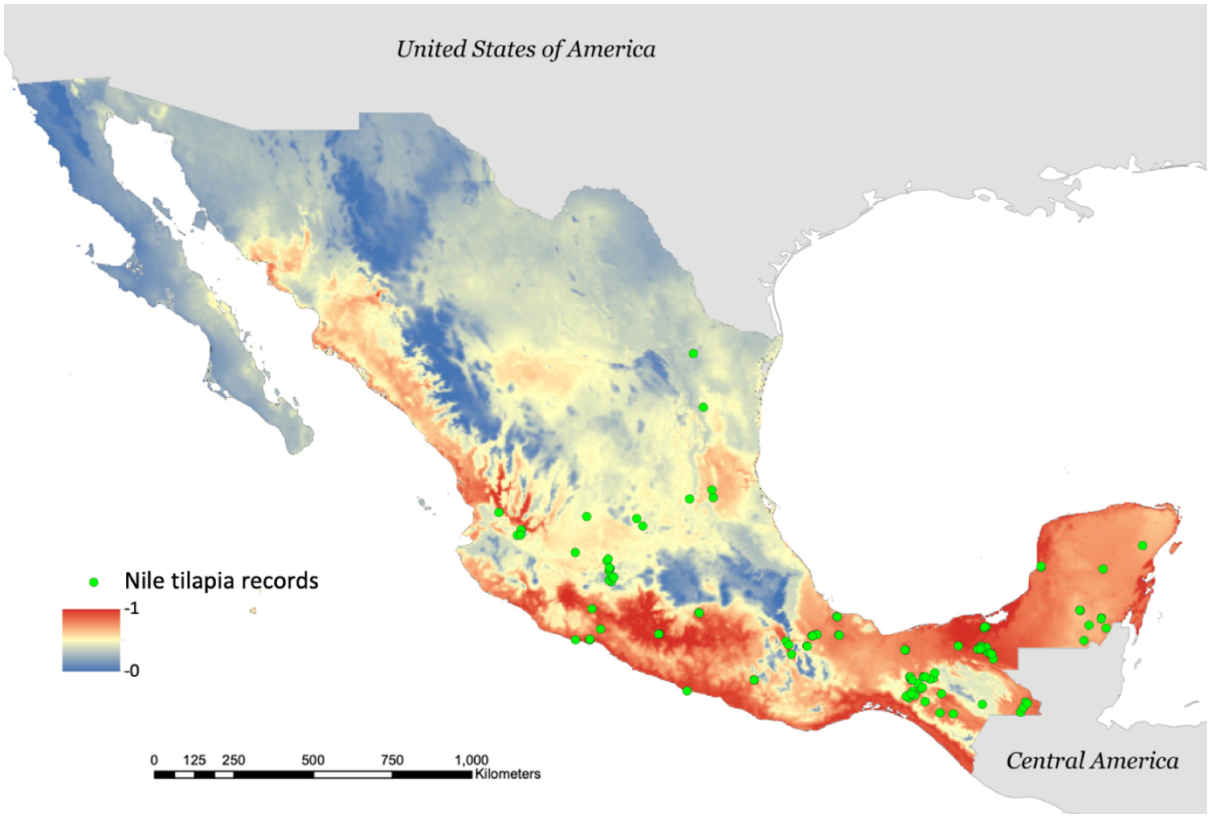



**Figure 1.4.** Modelled habitat suitability for Nile tilapia in Mexico, where red indicates regions with high suitability for the Nile tilapia, blue indicates regions with low suitability. Environmental data (annual mean temperature, maximum temperature of warmest month, minimum temperature of coldest month) for the period 1960 to 1990 are from Worldclim (Fick & Hijmans 2017). The model was generated using Maxent (Phillips *et al.* 2004), using Nile tilapia occurrence records downloaded from GBIF (https://www.gbif.org/) on 30 June 2019 (https://doi.org/10.15468/dl.11pyhd).

Some of the most substantive impacts of invasive species may be on closest related native species, due to their ecological similarities, aggressiveness and overlapping niches (Sanchez *et al*. 2012). At least 70 native fish species are known from Mexico (Miller *et al.* 2009; Froese & Pauly 2019), many of which are local or regional endemics, and share similar habitat use patterns to tilapia species. Hence, it is valuable to assess how Nile tilapia overlaps with native

biodiversity. The area with the highest cichlid richness is in the southeast of the country between Tehuantepec Isthmus (South of Veracruz), Pantanos de Centla (Tabasco) and Laguna de Términos (Campeche), with up to 13 species present. This area is proximate to the Yucatan peninsula, where between six to nine species are typically present locally (Figure 1.5). Notably, this area of highest cichlid species richness corresponds with a high probability of presence of Nile tilapia (Figure 1.4).

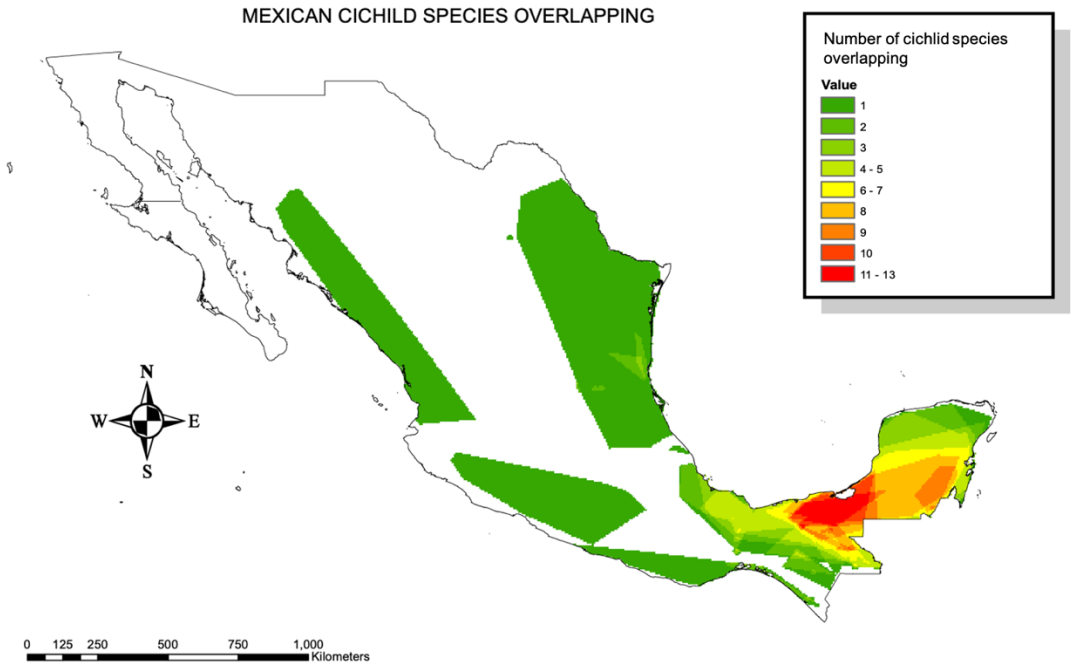



**Figure 1.5.** Composite map of cichlid species richness across Mexico, based on maps of 48 species in Miller *et al.* (2009). The maps were digitised and georeferenced, a matrix with the georeferenced distribution sites was built and used to construct a distribution polygon per species. These polygons were then overlaid to generate the composite biodiversity map. All the steps were performed using different functions in ArcMAP, version 10.5.1, (ESRI, 2013).

The ecoregions with the highest cichlid species richness tend to be the Yucatan dry and moist forest habitats, including the mesoamerican Gulf-Caribbean mangroves, Pantanos de Centla, and Peten-Veracruz moist forests (Figure 1.6).

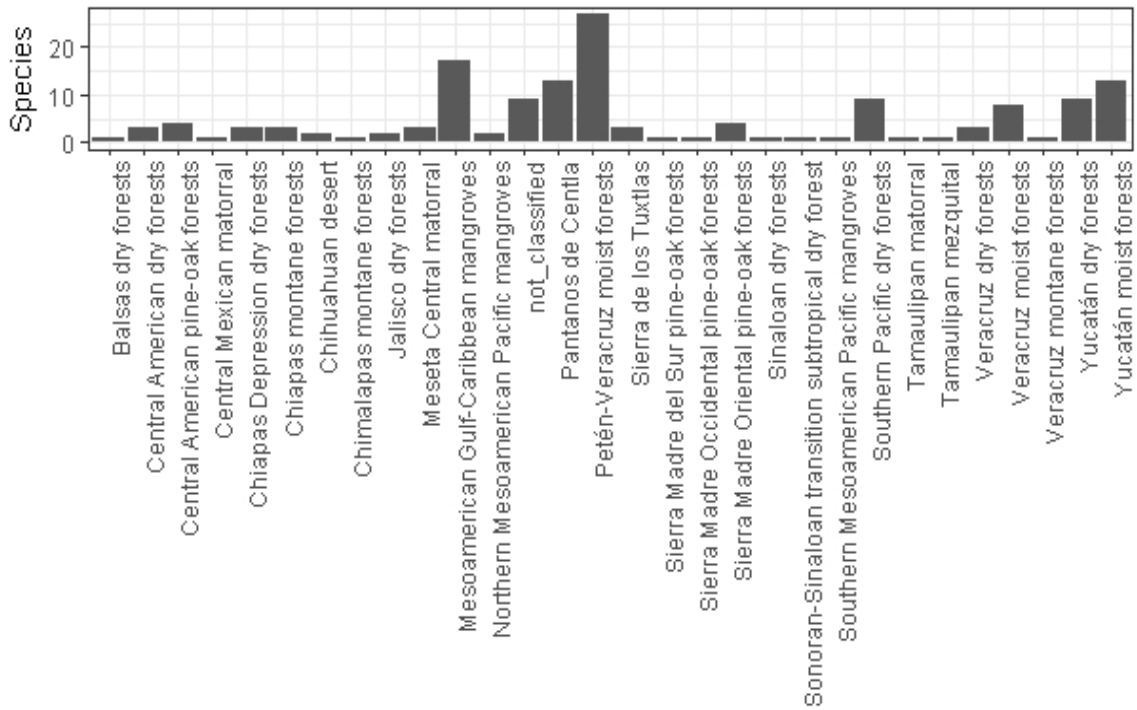



**Figure 1.6** Ecoregions with the highest species richness of native cichlid species in Mexico. Occurrence records for 46 species were sourced from GBIF (https://www.gbif.org/), and linked to World Wildlife Fund Bioregions (Olson *et al.,* 2001), using the Stacked Species Distribution Modelling package in R (Schmitt *et al.* 2017).

### 1.11 Studying the distribution of species using environmental DNA (eDNA)

Conventional methods of studying fish species distributions and habitat use require direct evidence of species presence, from capture or photographic records. However, over recent

years the utility of environmental DNA (eDNA) as a tool to study species distributions is starting to be realised. Environmental DNA is typically thought of as DNA released into the environment from faeces, urine, skin shedding and other natural processes. This material can be sampled from environmental sources including soil, water or air (Taberlet *et al.* 2012). Once in the environment, eDNA preservation and availability vary from a few days at elevated temperatures in tropical ecosystems (Eichmiller *et al.* 2016; Robson *et al.* 2016), to weeks in temperate freshwater systems (Dejean *et al.* 2011; Thomsen *et al.* 2012a). It may even persist for hundreds of thousands of years in cold and dry permafrost (Willerslev *et al.* 2007). The fundamental principle of eDNA analyses is that the sequences of this DNA from the environment can be matched to available databases to enable taxonomic or functional information to be gained for the ecosystem under consideration (Taberlet *et al.* 2018).

The methods used to analyse eDNA have their origins in the field of microbiology during the 1980s and 1990s, where DNA was used to describe microbial communities in soil, aquatic sediments and water (Diaz-Ferguson & Moyer 2014). In contemporary work, environmental DNA can be analysed using multiple methods, with the most commonly applied methods being: i) high-throughput shotgun sequencing of bulk DNA in samples; ii) high-throughput sequencing of DNA fragments amplified using “metabarcode” PCR methods, typically targeting multiple taxa within samples; and iii) targeted analysis of specific-species DNA fragments using real-time (quantitative) PCR primers and probes. The reliability of these methods has increased over recent years, and now the laboratory and computational procedures enable, in principle, rapid generation of data and reduced economic spend relative to conventional survey methods (Ji *et al.* 2013).

Environmental DNA methods are now commonly used to address research questions in several fields, including conservation biology, fundamental ecology and palaeontology. These methods have contributed new biodiversity discoveries, and new records of species that are difficult or impossible to survey using traditional ecological methods (Goldberg *et al.* 2015; Stat *et al.* 2019; Valdez-Moreno *et al.* 2019). Applications including the assessment and monitoring of endangered and invasive species have contributed to conservation actions (Thomsen *et al.* 2012b; Stoeckle *et al.* 2016; Klymus *et al.* 2017; Valdez-Moreno *et al.* 2019). However, as it stands the use of eDNA is biased in both taxonomic and habitat coverage. The majority (84%) of eDNA research tends to focus on the study of fish and amphibians, and the most surveyed ecosystems are freshwater systems (61%), while oceanic systems are less commonly studied (5%). The remainder (34%) are mesocosm studies, often describing tests of eDNA methodology (Roussel *et al.* 2015).

**1.12 Benefits and limitations of the use of eDNA.**

One of the major benefits of eDNA-based analyses is that they enable non-invasive monitoring of elusive threatened species, such as the Yangtze finless porpoise (*Neophacena asiaeorientalis*) in China (Qu & Stewart 2019), the great crested newt (*Triturus cristatus)* in the UK (Rees *et al.* 2014), or the stonefly *Isogenus nubecula* in Wales (Mauvisseau *et al.* 2019). Typically, these analyses are undertaken using species-specific primers and probes, that enable the quantification of the number of target eDNA copies in the sample. Whole community analyses using eDNA metabarcoding methods can also reveal biodiversity of species. For example, eDNA metabarcode analyses have revealed the presence of the invasive suckermouth catfish *Hypostomus plecostomus* in the Rio Hondo and Bacalar Lake in Quintana

Roo, Mexico (Valdez-Moreno *et al.* 2019). Where tested, the relative number of metabarcode reads assigned to species does tend to positively correlate with known abundance in both experimental trials (Klymus *et al.* 2017) and there are often positive associations between eDNA metabarcode read abundance and conventional survey data (Lamb *et al.* 2019). For example, eDNA metabarcode reads of stream amphibians in the Brazilian Atlantic Forest correlated positively with known abundance from traditional survey methods (Sasso *et al.,* 2017).

There are multiple issues to consider when designing and interpreting results from eDNA studies. One issue it that eDNA can frequently result in the occurrence of false negative results (Goldberg *et al.* 2016). It has been frequently shown in comparisons between eDNA traditional survey methods, such as the fish communities of the Hokkaido Lakes in Japan where only 70% of known species diversity was recovered using eDNA (Fujii *et al.* 2019), despite the 12S fish metabarcoding approach used being generally considered robust and reliable (Collins *et al.* 2019). There are multiple potential reasons for the failure of eDNA metabarcoding to detect species. It may be that primer combinations have intrinsic mismatches with primer sequences, or that the presence of inhibitors in the sampled water lead to failures of PCR. Alternatively, it could be that some species in communities have fundamentally low eDNA release rates. For example, in a seasonal survey of invasive fish species in Australia, the number of reads for oriental weather loach (*Misgurnus anguillicaudatus*) and red fin perch (*Perca fluviatilis*) had a positive association with Catch Per Unit Effort from a conventional survey. By contrast, common carp (*Cyprinus carpio*) showed a negative association, even falsely appearing as absent autumn eDNA read data, and showing meagre numbers of reads in spring (Hinlo *et al.* 2017).

Another major issue is the persistence rate of DNA, and whether the presence of eDNA reads can be used to reliably infer the local and recent presence of species. Studies of decay rates have typically revealed that eDNA decays at exponential rates in aquatic systems, with the highest rates of detection present within the first 48 hours since release. However, the rate of decay has been shown to be dependent on the multiple environmental factors, with faster rates of decay in freshwaters being particularly associated with higher temperatures (Lance *et al.* 2017; Tsuji *et al.* 2017). Nevertheless, despite eDNA degradation being faster in tropical conditions, eDNA has been found to reliably reveal the diversity of species in tropical environments (Cantera *et al.* 2019; Cilleros *et al.* 2019; Ivanova *et al.* 2019).

A further issue to consider with interpretation of metabarcode read data is whether false negatives occur because of databases used to evaluate read identity are incomplete. Open-access databases including genbank (https://www.ncbi.nlm.nih.gov/genbank/) and the Barcode of Life (http://www.boldsystems.org/) are available but can be limited in taxonomic coverage for particular species groups and regions of the world. Hence, bespoke databases for taxa of interest may need to be generated in parallel with the eDNA studies.

### 1.13 Knowledge gaps relating to tilapia invasion in Mexico

At present, although there is good information on the taxonomic diversity of Mexico's freshwaters, and core information on the distribution of species, there remains relatively little understanding of environmental drivers of biodiversity, and thus a fundamental understanding of the relative abundance of species in relation to environmental variables may help to better understand the spatial distribution of species across the country. To date, the utility of environmental DNA to quantify this diversity is largely unexplored. At present

the only application of eDNA methods to resolve the presence of fish species of the country has been conducted in the area of Lake Bacalar in the Yucatan Peninsula, using metabarcoding of the COI barcoding gene (Valdez-Moreno *et al.* 2019). While this study revealed the presence of a high diversity of species, it was unclear if eDNA read abundance correlated with abundance measured using conventional capture-based survey methods, or was able to recover the presence of invasive species.

There are now applied reasons to better understand the structure of Mexico's freshwater fish communities. There is now strong evidence to suggest that Nile tilapia, in particular, has a wide range in the country. This range may increase as tilapia aquaculture expands due to positive impacts on the economy of rural areas in Mexico, and also the sponsorship by the national government and private corporations. Currently there is limited knowledge of Nile tilapia niche use relative to native fish species in the natural environment, or their impacts on native species. The availability of such knowledge could help to promote the sustainable development of the industry, for example by encouraging the production facilities to incorporate management mechanisms that avoid accidental releases, and therefore avoid potential negative impacts of invasive species in native environments. At the national level, such core information would also enable organisations such as Mexico's autonomous Council for the Knowledge and Use of Biodiversity (CONABIO) to promote policies for the management of invasive species. Already this Council has a set of defined guidelines that could help to alleviate the situation with invasive species in the country (Koleff *et al.* 2010).

### 1.14 Aims and scope of the thesis

Chapter 2 of this thesis addresses the question of whether Nile tilapia is able to dominate native Mexican cichlid species in competition for space. Behavioural interactions between Nile tilapia and the indigenous Mayan cichlid (*Mayaheros urophthalmus*) are quantified under experimental conditions, and water temperature, pH and oxygen are tested as environmental mediators of behaviour. Chapter 3 of the thesis evaluates the potential for environmental DNA metabarcoding to describe the fish communities in Mexico's freshwaters, using Lake Caobas on the Yucatan peninsula as a study system. The lake was chosen as it contains a high diversity of native species and has been invaded by Nile tilapia. The outputs of eDNA analysis, from 12S metabarcoding methods, are compared to the results of a conventional capture-based survey method. Chapter 4 of the thesis reports a study of the ichthyofauna of six lakes in the state of Quintana Roo on the Yucatan Peninsula. The goals of the study are to evaluate the abundance of invasive *Oreochromis* within lakes, to test for environmental drivers of native fish biodiversity, and to investigate if that native biodiversity has been substantially influenced by the presence or absence of tilapia. The results of the research are summarised in Chapter 5 of the thesis. The results are discussed from the perspectives of conservation and management, and also used to direct suggestions for future research.

# Chapter 2

# Extreme environmental tolerance and competitive dominance favour invasive success of Nile tilapia

An adapted version of this chapter is in preparation to be submitted to a peer-reviewed journal:

Gracida Juarez C.A., Ioannou, C.C. and Genner, M.J. Extreme environmental tolerance and competitive dominance favour invasive success of Nile tilapia

Author contributions: CAGJ, CCI and MJG designed the study and conducted the first trials. CAGJ conducted the rest of the experiment and collected video-data. CAGJ, CCI and MJG conducted the statistical analyses. CAGJ led the writing of the manuscript. CCI and MJG contributed critically to the drafts.

**Abstract**

Invasive species have led to substantial changes in the biodiversity of freshwater ecosystems around the world. The African cichlid fish Nile tilapia (*Oreochromis niloticus*) is now one of most widely distributed of all freshwater species globally through its use in aquaculture and improvement of capture fisheries. Feral populations can readily establish and have been proposed to influence the diversity of native species through competitive effects. However, direct evidence of competition between Nile tilapia and native species is notably rare, and it is not clear how environmental variables such as temperature and oxygen may modulate competition. In this research, interactions were studied between Nile tilapia and the native Mayan cichlid (*Mayaheros urophthalmus*) in south-eastern Mexico. Mayan cichlid behaviour and space use were scored in experimental mesocosms varying in temperature, oxygen content and Nile tilapia density. It was found that Nile tilapia was by far the most active and aggressive of the two species, and their activity was only weakly influenced by temperature and oxygen concentration within the mesocosms. By contrast, the activity of the less aggressive Mayan cichlid was strongly predicted by the activity and aggressive behaviour of the Nile tilapia, and Mayan cichlids showed a steep decline in behaviours with increased water temperature and reduced oxygen. These results provide evidence that the broad environmental tolerance of the intrinsically aggressive Nile tilapia enables it to perform more effectively than native species in challenging conditions. This suggests that Nile tilapia may have an advantage over native species during periods of extreme conditions, which may help to exacerbate its invasive success as those conditions become more frequent in a changing world.

## 2.1 Introduction

Invasive species are currently considered to be one of the major drivers of contemporary biodiversity loss. They can act to erode native species diversity directly through processes including predation (Doherty *et al.* 2016), competition (Gurevitch & Padilla 2004) or pathogen transmission (Gozlan *et al.* 2005), but can also affect native species indirectly by changing the distribution and availability of key ecological resources, including food and breeding habitats (Cuddington & Hastings 2004). In aquatic systems there are many cases where invasions that have caused substantial changes to natural ecosystem structure, including degradation of trophic interactions and water chemistry (Vitousek *et al.* 1997). Invasions have in some circumstances driven considerable economic loss to industrial sectors including fisheries based on indigenous species, power generation and shipping (Lovell *et al.* 2006).

Over recent years much of the research on aquatic invasive species has focused on identification of traits associated with invasion success of species (García-Berthou 2007). This has highlighted a suite of characteristics that invasive species tend to possess, including high growth rate, short generation time, prolific reproduction, small propagule sizes, a good ability to resist enemies, broad dispersal, high competitive ability, and a wide environmental tolerance (García-Berthou 2007; Whitney & Gabler 2008). Importantly however, there has been less focus on research to identify precise ecological mechanisms by which invasive species affect their invaded ecosystems. This is of importance because knowledge of ecological mechanisms can help us to predict the consequences of future invasions (e.g. Kiesecker *et al.* 2001; Kiruba-Sankar *et al.* 2018). A mechanistic understanding of the consequence of invasions is not necessarily straightforward to achieve and may require combined information from both field observations and experimentation in controlled

conditions. For example, in cases where an increased abundance of an invasive species is paralleled by decline in a co-occurring native species, competition should only be inferred if species are shown to share limited resources, and negative effects of the invasive species on the native species are present in experimental circumstances (Didham *et al.* 2005).

A further advantage of understanding mechanisms by which invasive species contribute to ecological changes in non-native systems is that it may be possible to determine how the outcomes of biological invasions are influenced by changing environments. Specifically, in the case of interspecific competition between invasive and indigenous species, the outcome may be determined by their relative performance across environmental gradients (Carmona-Catot et al. 2013). Moreover, it is also possible that outcomes are dependent upon to synergistic interactions between the environmental characteristics. Of particular concern is that climate change may provide novel environments where invasive species are able to dominate native species, because of they possess broader physiological tolerances (Bates *et al.* 2013).

One of most widely introduced species globally is Nile tilapia (*Oreochromis niloticus*), which is native to northern and eastern Africa and has been introduced into more than 100 countries worldwide for aquaculture and improvement of capture fisheries (Anuario 2016). A broad environmental tolerance and relatively fast growth has favoured use of the species in food production systems, but also has enabled it to establish feral populations in natural water bodies (Canonico *et al.* 2005). It has been reported that Nile tilapia compete actively and aggressively for resources with ecologically-similar native species, causing rapid decreases of resident populations (Canonico *et al.* 2005). However, to date the experimental evidence for competition between Nile tilapia and other species is limited to a small number of studies. Specifically, Martin *et al*. (2010) demonstrated, in laboratory aquaria, that juvenile Nile tilapia

were able to outcompete size-matched juvenile red-spotted sunfish (*Lepomis miniatus*) over preferred shelter, elevating predation risk for the displaced sunfish. Meanwhile, in experimental aquaculture ponds Ahmad *et al.* (2010) showed that the presence of Nile tilapia resulted in lower biomass yields of three cyprinid species.

In Mexico, Nile tilapia is now widespread in natural water bodies, as a consequence of deliberate introductions to enhance fisheries productivity and through accidental escapes from commercial aquaculture facilities. The species also has a broad spatial range of suitable habitat in the country, making it likely that the expansion will continue (Figure 2.1). However, it is unclear if there is potential for the species to undergo interference competition with indigenous species for limited resources. Due to the history of rapid establishment in Mexican freshwater systems and the physiological features that have favoured its spread in the wild, it is assumed that the Nile tilapia is succeeding similarly in south-eastern Mexico. Thus, this study addresses the question of whether Nile tilapia is able to dominate the native Mayan cichlid (*Mayaheros urophthalmus)* in competition for space under experimental conditions. It was hypothesised that since *Oreochomis niloticus* has been demonstrated to aggressive exclude sunfish from shelter, it would be able similarly exclude cichlid fishes using a similar resource. Additionally, given the possibility that environmental conditions may influence space use and behaviour, the effects of water temperature, pH and oxygen were investigated as environmental mediators of behaviour. It was hypothesised that the broad environmental tolerances of Nile tilapia would ensure that changing environment had only a marginal effect on its behaviour, while the Mayan cichlid would show stronger responses to changing conditions.

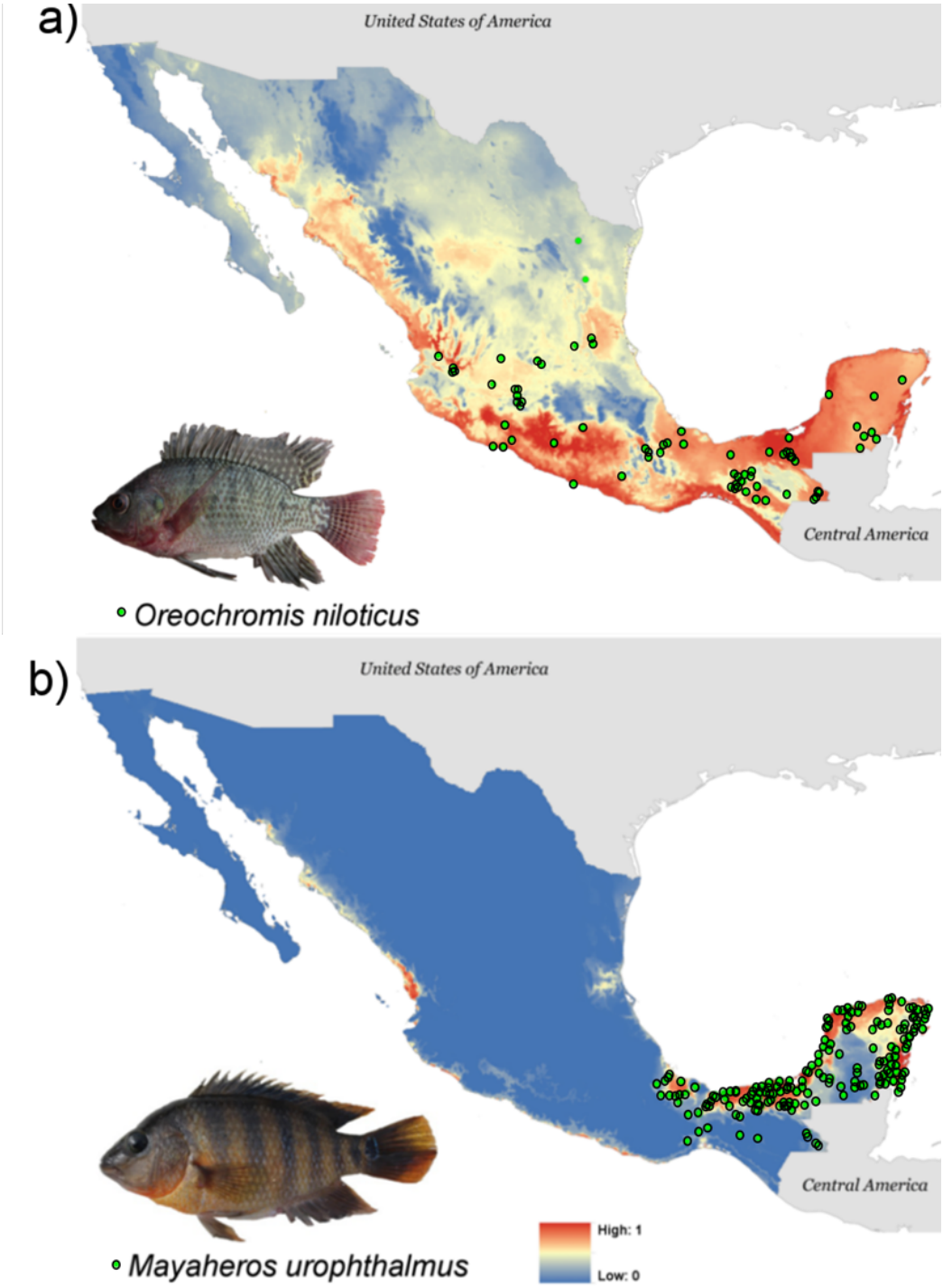



**Figure 2.1.** Habitat suitability of the a) Nile tilapia and (b) Mayan cichlid. Occurrence records (green circles) are derived from GBIF (Global Biodiversity Information Facility), while habitat suitability is modelled using Maxent (Phillips *et al.* 2004) using environmental data (annual mean temperature, maximum temperature of the warmest month, minimum temperature of the coldest month) across the period 1960 to 1990 from Worldclim (Fick & Hijmans 2017).

## 2.2 Material and Methods

### *2.2.1 Study system*

The study took place in the state of Quintana Roo on the Yucatan Peninsula. The federal office for fisheries at Quintana Roo (as cited in Schmitter-Soto & Caro 1997) reported that since the 1970's multiple tilapia farms have been established in the state, although production was low in the early 1990's (9.5 tonnes per annum, 0.1% of the national output), and remained low as recently as 2010 (Mexico Government 2012). More recently, however, tilapia production in the region has overcome a time of uncertainty associated with the elevated costs of aquaculture feed supply and electricity, both essential for the tilapia enterprise success (Castillo 2018). Recently, Quintana Roo authorities have estimated tilapia production surpassed 100 tonnes of 2017, and increased to 300 tonnes in 2018 (Zamora 2018). During 2019 the tilapia producers of Quintana Roo, in association with the University of Alabama, adapted tilapia production technology to further reduce production costs. With these modifications, they have estimated that by the end of 2020 the production will increase to 500 tonnes annually, and this could continue to increase to 1000 tonnes by 2022 (Castillo, 2019).

Nevertheless, despite the low production, *Oreochromis* are now widely established in natural water bodies, with relative abundances as high as 20% of number of individual fish sampled (Schmitter-Soto & Caro 1997), with Nile tilapia in particular being present in both aquaculture and invaded freshwater habitats. Commonly found alongside Nile tilapia is the Mayan cichlid, a species endemic to Atlantic-slope drainages of Central America, and with a much narrower range than Nile tilapia (Figure 2.1). Nevertheless, the species is tolerant of high salinities, occupying freshwater, estuaries and coastal marine environments within this native range. It

is also in itself an invasive species where it has been introduced to the south-eastern USA (Schofield *et al.* 2010). The two species grow to similar sizes in Central America but differ considerably in dietary preferences. Nile tilapia is primarily a planktivore and detritivore, while Mayan cichlid is a benthic predator of fish and macroinvertebrates. Nevertheless, the two species do co-occur in habitats across Yucatan Peninsula, and use similar macrophyte-rich habitats as shelter providing the potential for competition between these two species.

### *2.2.2 Housing*

Nile tilapia were sourced from an aquaculture facility located in Noh-Bec, Quintana Roo. Transport to the experimental site took place in a 1000 L water container filled up to 500 L. Air was pumped through the water during transportation. The Instituto Tecnologico Superior de Felipe Carrillo Puerto provided Mayan cichlid individuals for the experiment that had been bred in semi-natural conditions on site. Once on site, fish were placed in single species holding ponds each with ~5000 L of water. Once in the ponds, fish specimens were held for at least two days, acclimatizing and under observation to ensure there were no signs of poor health before starting experimental trials.

### *2.2.3 Experimental set up*

All experiments took place in a set of circular inflatable pools (mesocosms), each measuring 138 cm in diameter and 30 cm depth. These were positioned in a row of four, in a shaded outdoor environment at the Instituto Tecnologico Superior de Felipe Carrillo Puerto. There were two experimental treatments. A low tilapia density treatment had five Mayan cichlids per mesocosm and five Nile tilapia (n = 31), while a high tilapia density treatment had five Mayan cichlids and 10 Nile tilapia per mesocosm (n = 33). The specific densities were not

based on any *a-priori* evidence, but rather the hypothesis that a greater density would intensify any interspecific competition present. The 64 experimental replicates were conducted in 16 experimental time blocks, during which four experimental replicates were running at once. Mayan cichlids used in the experiment were larger than the Nile tilapia (Mean total length; Mayan cichlid = 11.87 cm ± 1.16 SD; Nile tilapia = 10.2 cm ± 0.75 SD).

Each experimental pool was filled with clean water from a groundwater pump to a depth of 20 cm. The bottom of each pool was covered with a layer of gravel. Each pool had a focal 'shelter area', delimited by vertical lines drawn on the walls of the pool (Figure 2.2), which contained a clump of aquatic macrophytes (*Eichhornia crassipes*) of similar dimensions and biomass across the replicate pools. Prior to the start of each trial, a HERO5 (GoPro, CA) camera on a tripod was placed on the opposite side of the pool to the shelter, enabling the video recording of the shelter area. The settings of each camera (720 pixels, 120 frames per second, wide field of view) provided a suitable resolution for later analyses. After the camera was in place, fish specimens to use during the trial were captured from the source pond using a 3 m in diameter cast net and immediately placed in a 20 L container, from which were taken individually using an aquarium net. The total length of each fish was then individually measured and released into each experimental pool. The fish species were released in a random order into each pond and allowed to acclimatize for 10 minutes before filming for 30 minutes. After filming, water temperature and pH were measured using a pH-meter (ExStik™, China), while dissolved oxygen was measured using a portable DO-meter (HI-9146 Handheld Dissolved Oxygen Meter, Hanna Instruments, Romania). Each fish was used only once, and after the experiment fish were placed in different single-species holding ponds to the unused fish.

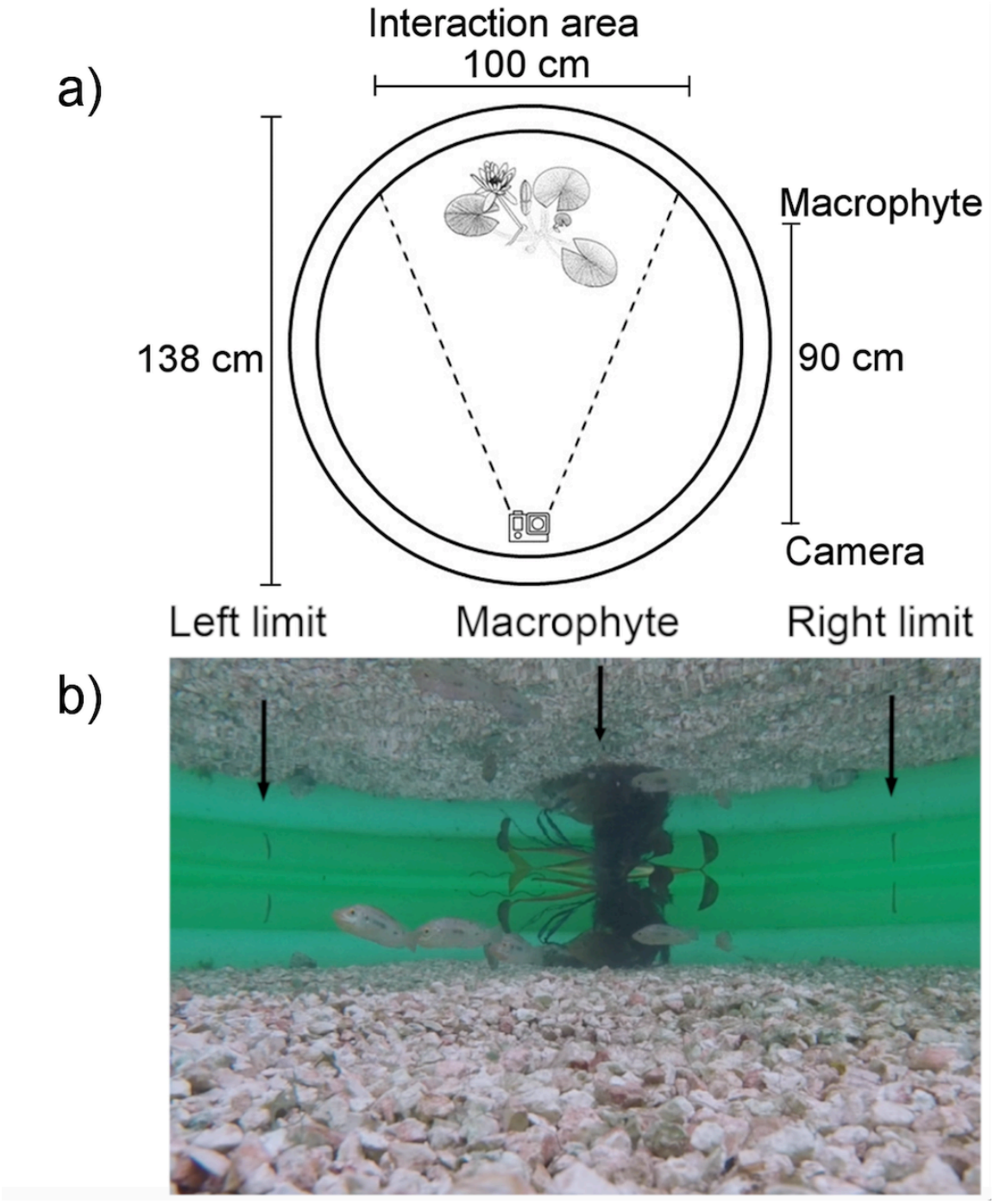



**Figure 2.2.** Experiment set up. a) Layout of the experimental mesocosms, b) Observation arena of the space filmed during the experiment. Either side of the macrophytes, two vertical lines delineate the shelter area.

### *2.2.4 Experimental Data analysis*

For the video analysis of behaviour, the software BORIS (Behavioural Observation Research Interactive Software) was used (Friard & Gamba, 2016), for which a custom ethogram, associating keys to point events of interest (Supplementary Information 2.2) was generated. The behaviours scored were designed to enable the calculation of metrics of aggressive interspecific interactions, the movement activity of each species, and the average time spent by each species in the shelter area. The behavioural events were scored for a 15-minute period from each video (minutes 3 to 18). BORIS software allows recording all events of a video in an exportable data matrix for further analysis.

For each experimental replicate, the total activity (= activity) was calculated separately for each species as the total number of occasions when individuals of each species left the shelter area (by crossing one of the vertical lines). In addition the total number of aggression events (= chases initiated) initiated by Nile tilapia directed at Mayan cichlids was calculated, and the total number of aggression events initiated by Mayan cichlids directed at Nile tilapia. The average number of individuals in the shelter across the whole 15-minute observation period (= shelter use) was calculated using the time points when fish left and returned to the shelter area.

For the trials where Mayan cichlids and Nile tilapia were in the same densities, the frequencies of behaviours between species were compared using Wilcoxon matched-pair Signed-Rank tests. To compare the behaviour of Mayan cichlids in different Nile tilapia densities, Mann-Whitney U-tests were used. To determine the main factors influencing the behaviour of each species linear models were constructed in R 3.6.1 (R Core Team, 2019). Each behavioural response variable (activity, chases initiated, shelter use) for each species was modelled

separately, resulting in six linear models. For predictor variables, the measured environmental variables (pH, dissolved oxygen, temperature) were included, alongside the three behavioural variables of the heterospecific species (activity, chases initiated, shelter use), and the categorical variable of the experimental pool identity. Due to the slightly unbalanced nature of the data (31 same density trials and 33 dissimilar density trials), a type II regression model was used, using the R package car (Fox & Weisberg, 2019).

## 2.3 Results

### *2.3.1 Comparisons of behaviour between Nile tilapia and Mayan cichlid equal density treatments*

In trials where both species were represented by five individuals, Nile tilapia were significantly more active than Mayan cichlids (Wilcoxon test, $Z = -4.782$, $P < 0.001$; Figure 2.3a). Nile tilapia were also significantly more aggressive than Mayan cichlids (Wilcoxon test, $Z = -3.466$, $P < 0.001$; Figure 2.3b), and the Nile tilapia spent significant less time in shelter (Wilcoxon test, $Z = -4.321$, $P < 0.001$; Figure 2.3c).

### *2.3.2 Comparisons of Mayan cichlid behaviour in different densities of Nile tilapia*

In trials where Mayan cichlids were exposed to either five or ten individuals of Nile tilapia, it was found that Mayan cichlids were not significantly more active (Mann-Whitney test, $Z = -0.780$, $P = 0.435$; Figure 2.4a), not significantly more aggressive to heterospecifics (Mann-Whitney test, $Z = -0.705$, $P = 0.481$; Figure 2.4b), and did not spend significantly more time in shelter (Mann-Whitney test, $Z = -0.712$ , $P = 0.477$, Figure 2.4c).

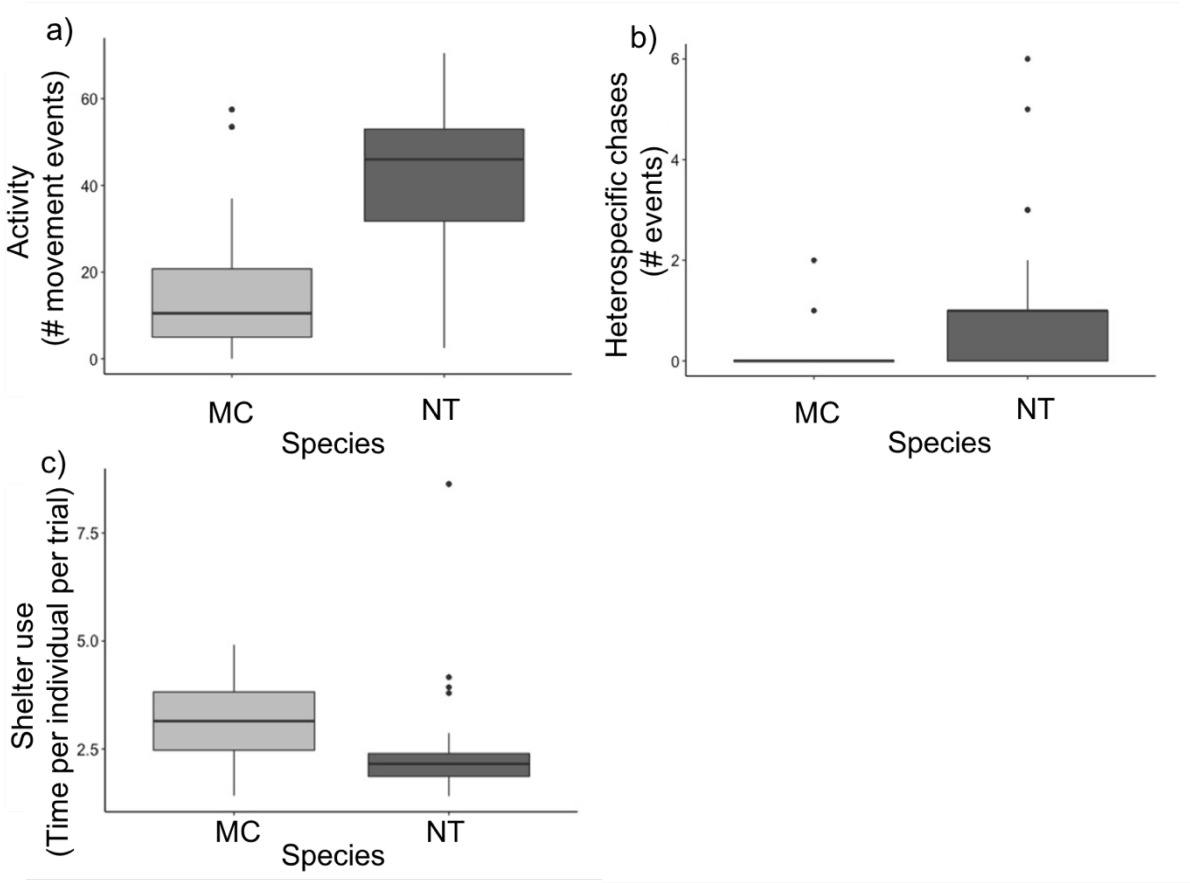



**Figure 2.3**. Differences between Nile tilapia (NT) and Mayan cichlid (MC) behaviour in the equivalent density treatment (5 individuals of each species) during the 15-minute observation periods. Boxplots show a) total activity, b) number of heterospecific chases initiated and c) average number of individuals in shelter. The median is represented by the thick horizontal line, the interquartile range is enclosed in the box, the whiskers extend to the most extreme data points within 1.5 × the interquartile range outside the box, and the dots show data points beyond the whiskers.

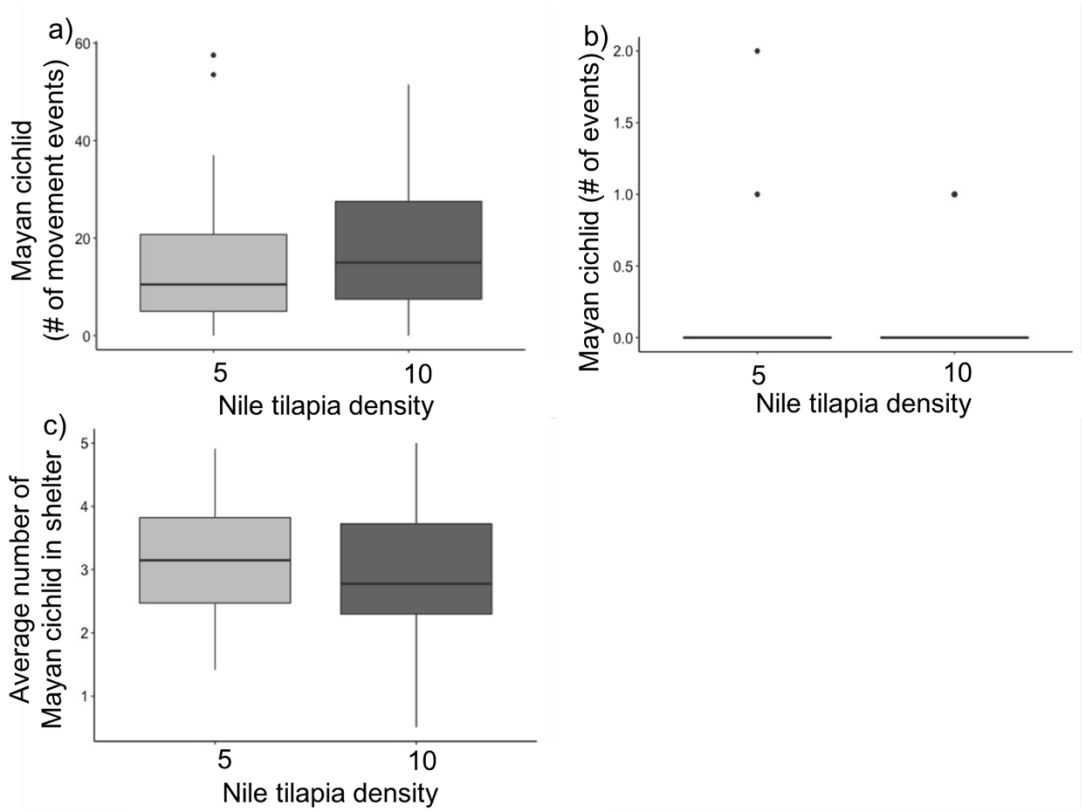



**Figure 2.4**. Mayan cichlid behaviour in differing densities of Nile tilapia (5 or 10 individuals) during the 15-minute observation periods. Boxplots show a) total Mayan cichlid activity, b) number of heterospecific chases initiated by Mayan cichlid and c) average number of Mayan cichlid individuals in shelter.

### 2.3.3 Variables associated with Mayan cichlid behaviour

The total activity of the Mayan cichlids was significantly related to Nile tilapia activity, the water temperature, dissolved oxygen concentration and the pH of the water (Table 2.1). Increases in Mayan cichlid activity were associated with more Nile tilapia activity (Figure 2.5), but the Mayan cichlid activity was reduced in warm temperature and low oxygen conditions (Figure 2.5). Mayan cichlid individuals only chased Nile tilapia on seven occasions in the total observed time, and there were no significant dependence of Mayan cichlid chases initiated

on any measured environmental variables (Table 2.1). Shelter use by Mayan cichlid individuals was also not significantly associated with any measured environmental variables (Table 2.1).

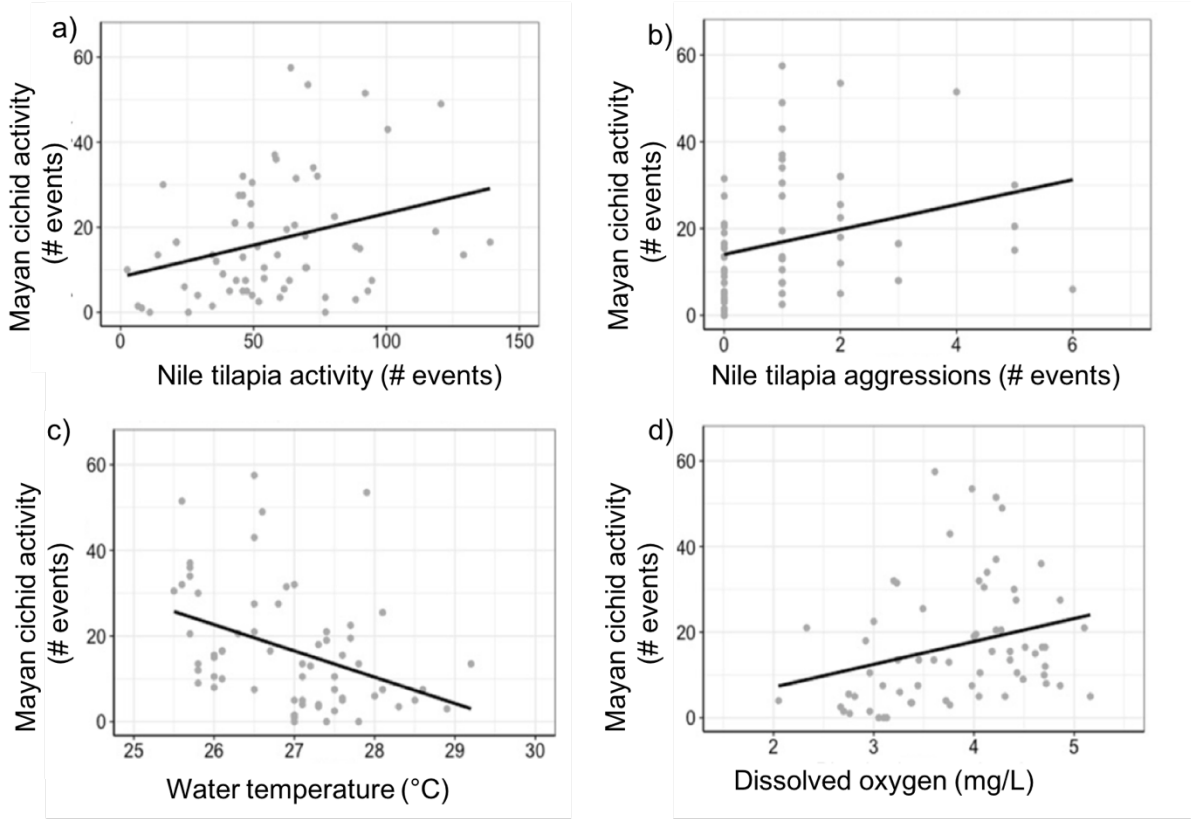



**Figure 2.5**. Associations between Mayan cichlid activity and a) total Nile tilapia activity, b) Nile tilapia aggression, c) temperature, d) oxygen content.

**Table 2.1** Predictor variables influencing the activity of the Mayan cichlid. Variables in bold font have statistically significant effects at $P < 0.05$.

| Measured variable | Predictor variables | *Df* | *SS* | *F* | *P* |
|---|---|---|---|---|---|
| Mayan cichlid activity | **Nile tilapia activity** | **1** | **1196.5** | **9.886** | **0.003** |
| | Nile tilapia density | 1 | 21.4 | 0.177 | 0.676 |
| | Nile tilapia chases initiated | 1 | 315.9 | 2.610 | 0.113 |
| | Nile tilapia in shelter | 1 | 122.3 | 1.011 | 0.320 |
| | Experimental pool | 3 | 540.5 | 1.489 | 0.231 |
| | **Dissolved oxygen** | **1** | **617.0** | **5.098** | **0.029** |
| | **pH** | **1** | **1269.3** | **10.487** | **0.002** |
| | **Temperature** | **1** | **1147.6** | **9.481** | **0.004** |
| | Nile tilapia activity * Nile tilapia density | 1 | 29.3 | 0.242 | 0.625 |
| | Nile tilapia chases initiated * Nile tilapia density | 1 | 77.4 | 0.640 | 0.428 |
| | Nile tilapia in shelter * Nile tilapia density | 1 | 13.4 | 0.110 | 0.741 |
| | Experimental pool * Nile tilapia density | 3 | 24.9 | 0.069 | 0.976 |
| | Dissolved oxygen * Nile tilapia density | 1 | 50.2 | 0.415 | 0.523 |
| | pH * Nile tilapia density | 1 | 70.2 | 0.580 | 0.451 |
| | Temperature * Nile tilapia density | 1 | 17.9 | 0.148 | 0.702 |
| | Residuals | 44 | 5325.6 | - | - |
| Mayan cichlid chases initiated | Nile tilapia activity | 1 | 0.000 | 0.002 | 0.963 |
| | Nile tilapia density | 1 | 0.049 | 0.346 | 0.559 |
| | Nile tilapia chases initiated | 1 | 0.203 | 1.436 | 0.237 |
| | Nile tilapia in shelter | 1 | 0.000 | 0.000 | 0.987 |
| | Experimental pool | 3 | 0.312 | 0.736 | 0.536 |
| | Dissolved oxygen | 1 | 0.006 | 0.042 | 0.840 |
| | pH | 1 | 0.219 | 1.550 | 0.220 |
| | Temperature | 1 | 0.016 | 0.110 | 0.742 |
| | Nile tilapia activity * Nile tilapia density | 1 | 0.514 | 3.635 | 0.063 |
| | Nile tilapia chases initiated * Nile tilapia density | 1 | 0.006 | 0.039 | 0.844 |
| | Nile tilapia in shelter * Nile tilapia density | 1 | 0.060 | 0.422 | 0.519 |
| | Experimental pool * Nile tilapia density | 3 | 0.114 | 0.268 | 0.848 |
| | Dissolved oxygen * Nile tilapia density | 1 | 0.124 | 0.875 | 0.355 |
| | pH * Nile tilapia density | 1 | 0.027 | 0.193 | 0.663 |
| | Temperature * Nile tilapia density | 1 | 0.015 | 0.107 | 0.745 |
| | Residuals | 44 | 6.220 | - | - |
| Mayan cichlid shelter use | Nile tilapia activity | 1 | 0.124 | 0.152 | 0.698 |
| | Nile tilapia density | 1 | 0.080 | 0.098 | 0.756 |
| | Nile tilapia chases initiated | 1 | 0.003 | 0.003 | 0.956 |
| | Nile tilapia in shelter | 1 | 0.106 | 0.131 | 0.720 |
| | Experimental pool | 3 | 1.399 | 0.573 | 0.636 |
| | Dissolved oxygen | 1 | 0.005 | 0.007 | 0.935 |
| | pH | 1 | 1.385 | 1.701 | 0.199 |
| | Temperature | 1 | 1.181 | 1.451 | 0.235 |
| | Nile tilapia activity * Nile tilapia density | 1 | 0.792 | 0.972 | 0.330 |
| | Nile tilapia chases initiated * Nile tilapia density | 1 | 0.625 | 0.767 | 0.386 |
| | Nile tilapia in shelter * Nile tilapia density | 1 | 0.005 | 0.006 | 0.938 |
| | Experimental pool * Nile tilapia density | 3 | 4.242 | 1.737 | 0.173 |
| | Dissolved oxygen * Nile tilapia density | 1 | 0.401 | 0.493 | 0.486 |
| | pH * Nile tilapia density | 1 | 0.255 | 0.313 | 0.579 |
| | Temperature * Nile tilapia density | 1 | 0.042 | 0.051 | 0.822 |
| | Residuals | 44 | 35.826 | - | - |

Df = Degrees of freedom, SS = Sum of squares.

### *2.3.4 Variables associated with Nile tilapia behaviour*

The total activity of Nile tilapia recorded was primarily dependent on the density of Nile tilapia present (Table 2.2). Additionally, the activity of Nile tilapia increased with temperature, which was most clearly observed in trials of where 10 Nile tilapia were present (Table 2.2; Figure 2.6). Nile tilapia activity was positively associated with Mayan cichlid activity (Table 2.2). The total number of heterospecific chases initiated by Nile tilapia was found to be significantly positively related to pH (Table 2.2). The number of individual Nile tilapia in shelter was only predicted by the density of Nile tilapia present in the trial (Table 2.2).

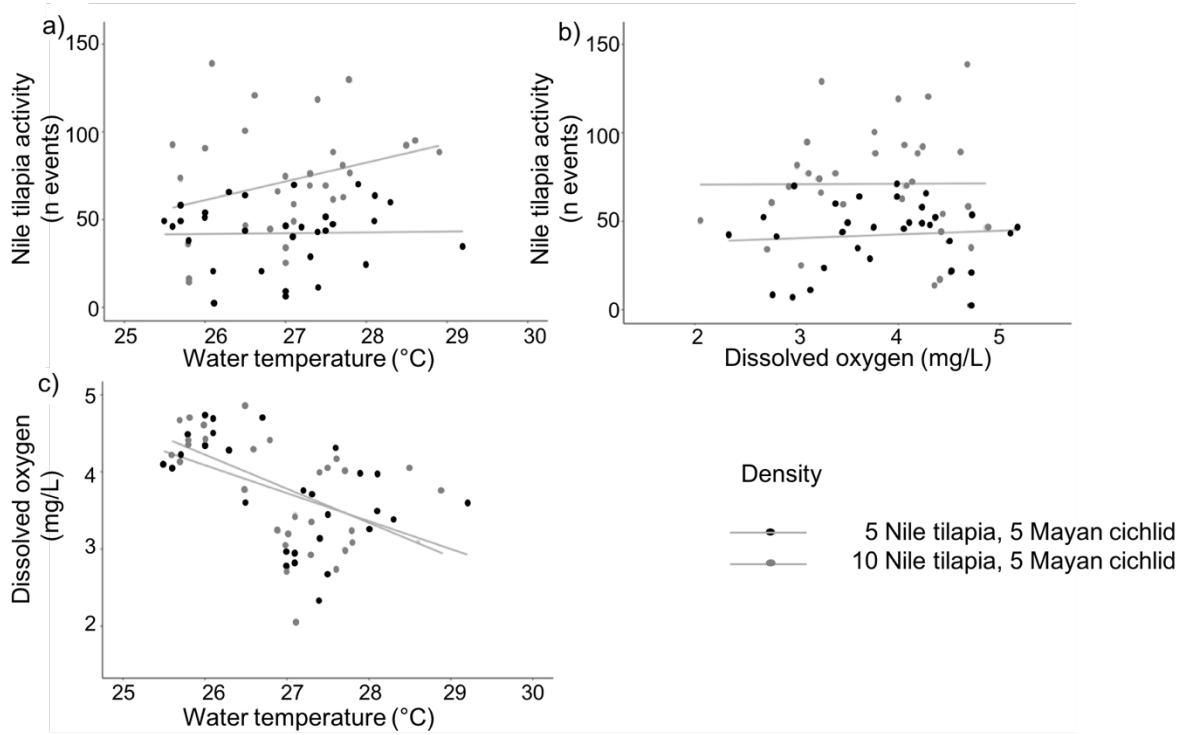



**Figure 2.6**. Associations between Nile tilapia activity and a) water temperature in high- and low-density treatments, b) oxygen content in high- and low-density treatments. Also shown is the relationship between oxygen content and water temperature in high- and low-density treatments (c).

**Table 2.2** Predictor variables influencing the activity of Nile tilapia. Variables in bold font have statistically significant effects at $P < 0.05$.

| Measured variable | Predictor variables | *Df* | *SS* | *F* | *P* |
|---|---|---|---|---|---|
| Nile tilapia activity | **Mayan cichlid activity** | **1** | **7514.3** | **13.748** | **0.001** |
| | **Nile tilapia density** | **1** | **9218.3** | **16.866** | **< 0.001** |
| | Mayan cichlid chases initiated | 1 | 233.1 | 0.427 | 0.517 |
| | Mayan cichlid in shelter | 1 | 767.4 | 1.404 | 0.242 |
| | Experimental pool | 3 | 1378.2 | 0.841 | 0.479 |
| | Dissolved oxygen | 1 | 1441.9 | 2.638 | 0.111 |
| | pH | 1 | 125.3 | 0.229 | 0.634 |
| | **Temperature** | **1** | **6192.5** | **11.330** | **0.002** |
| | Mayan cichlid activity * Nile tilapia density | 1 | 1031.2 | 1.887 | 0.177 |
| | Mayan cichlid chases initiated * Nile tilapia | 1 | 385.1 | 0.705 | 0.406 |
| | Mayan cichlid in shelter * Nile tilapia density | 1 | 579.7 | 1.061 | 0.309 |
| | Experimental pool * Nile tilapia density | 3 | 499.3 | 0.305 | 0.822 |
| | Dissolved oxygen * Nile tilapia density | 1 | 216.8 | 0.397 | 0.532 |
| | pH * Nile tilapia density | 1 | 19.3 | 0.035 | 0.852 |
| | Temperature * Nile tilapia density | 1 | 2201.4 | 4.028 | 0.051 |
| | Residuals | 44 | 24048.6 | | |
| Nile tilapia chases initiated | Mayan cichlid activity | 1 | 7.2 | 4.002 | 0.052 |
| | Nile tilapia density | 1 | 0.8 | 0.443 | 0.509 |
| | Mayan cichlid chases initiated | 1 | 0.5 | 0.262 | 0.611 |
| | Mayan cichlid in shelter | 1 | 1.5 | 0.851 | 0.361 |
| | Experimental pool | 3 | 14.6 | 2.729 | 0.055 |
| | Dissolved oxygen | 1 | 4.3 | 2.406 | 0.128 |
| | **pH** | **1** | **10.5** | **5.866** | **0.020** |
| | Temperature | 1 | 0.1 | 0.068 | 0.795 |
| | Mayan cichlid activity * Nile tilapia density | 1 | 0.0 | 0.016 | 0.899 |
| | Mayan cichlid chases initiated * Nile tilapia | 1 | 0.6 | 0.308 | 0.582 |
| | Mayan cichlid in shelter * Nile tilapia density | 1 | 1.1 | 0.594 | 0.445 |
| | Experimental pool * Nile tilapia density | 3 | 8.8 | 1.645 | 0.193 |
| | Dissolved oxygen * Nile tilapia density | 1 | 0.3 | 0.162 | 0.690 |
| | pH * Nile tilapia density | 1 | 0.4 | 0.235 | 0.631 |
| | Temperature * Nile tilapia density | 1 | 3.0 | 1.681 | 0.202 |
| | Residuals | 44 | 78.7 | | |
| Nile tilapia shelter use | Mayan cichlid activity | 1 | 0.4 | 0.401 | 0.530 |
| | **Nile tilapia density** | **1** | **68.6** | **63.617** | **< 0.001** |
| | Mayan cichlid chases initiated | 1 | 0.1 | 0.046 | 0.831 |
| | Mayan cichlid in shelter | 1 | 0.0 | 0.003 | 0.958 |
| | Experimental pool | 3 | 5.6 | 1.744 | 0.172 |
| | Dissolved oxygen | 1 | 0.0 | 0.017 | 0.897 |
| | pH | 1 | 1.6 | 1.439 | 0.237 |
| | Temperature | 1 | 0.2 | 0.189 | 0.666 |
| | Mayan cichlid activity * Nile tilapia density | 1 | 1.0 | 0.898 | 0.349 |
| | Mayan cichlid chases initiated * Nile tilapia | 1 | 0.6 | 0.511 | 0.479 |
| | Mayan cichlid in shelter * Nile tilapia density | 1 | 0.1 | 0.104 | 0.749 |
| | Experimental pool * Nile tilapia density | 3 | 3.3 | 1.014 | 0.396 |
| | Dissolved oxygen * Nile tilapia density | 1 | 0.4 | 0.412 | 0.524 |
| | pH * Nile tilapia density | 1 | 1.6 | 1.449 | 0.235 |
| | Temperature * Nile tilapia density | 1 | 0.1 | 0.072 | 0.790 |
| | Residuals | 44 | 47.5 | | |

Df = Degrees of freedom, SS = Sum of squares.

## 2.4 Discussion

### *2.4.1 Competition between the species*

In the experimental trials, Nile tilapia was the most active of the two species and spent significantly less time in the macrophyte shelter environment than Mayan cichlids. These observations are consistent with Nile tilapia being more tolerant of open environments, perhaps linked to greater intrinsic boldness in the novel environment of the experiment. Boldness has been associated with interspecific differences in habitat patterns in other fish species, for example between threespine stickleback (*Gasterosteus aculeatus*) and ninespine sticklebacks (*Pungitius pungitius*) (Webster *et al.* 2009). Different habitat use patterns may also be related to different feeding ecology of the species. Nile tilapia tend to have a generalist diet that typically includes a high proportion of planktonic food items (e.g. Nijru *et al.,* 2004). By contrast the Mayan cichlid is a more specialist species feeding upon benthic macroinvertebrate or fish prey (Martinez-Palacios *et al.* 1993). Thus, the greater use of open water environments by Nile tilapia may facilitate filter feeding on phytoplankton, while Mayan cichlid tends to be a less-mobile predator of sheltered environments.

It was notable that the majority of aggressive interactions towards heterospecifics were initiated by Nile tilapia towards the Mayan cichlid. Similar patterns have been found in dyadic contests staged between similarly sized Nile tilapia and another Neotropical cichlid, the pearl cichlid *Geophagus brasiliensis* (Sanches *et al.* 2012). Typically, in behavioural trials of cichlid fishes, aggressive interactions take place during interference competition for territorial space, with the more aggressive individuals typically being the territory holder that maintains and defends the territory against intruders (e.g. Hess *et al.* 2016). However, in this study heterospecific aggression was mainly initiated by Nile tilapia that overall spent less time in

the territory (the shelter) than the Mayan cichlid. This pattern could be suggestive of the Mayan cichlid, at least when not being reproductively active as in this study, being a relatively benign non-aggressive species. However, it is possible that territorial breeding pairs of the Mayan cichlid would be considerably more aggressive when guarding eggs and juveniles. Equally, it is possible that the use of shelter by Nile tilapia is context dependent, with its use primarily for periods of elevated predation risk. Predation risk is known to be a strong modifier of the behaviour of territorial cichlid fishes (LaManna & Eason 2007), but further experiments would be required to evaluate the relative value of shelter to Nile tilapia in various ecological contexts.

The impact of Nile tilapia behaviour on populations of native fishes, such as Mayan cichlid, will ultimately depend on whether their behaviour can influence survivorship. Higher levels of aggression initiated by Nile tilapia was observed relative that observed by Mayan cichlid, but importantly no evidence was seen that Nile tilapia reduced the shelter use of Mayan cichlid individuals during the experimental trails. Therefore, it is currently unclear if Nile tilapia aggression alone could influence the survivorship of Mayan cichlid individuals. Only in one study, to our knowledge, has Nile tilapia has been shown to be able to reduce survivorship of native fish (Martin *et al.* 2010). In that study juvenile Nile tilapia were able to outcompete juvenile red-spotted sunfish by restricting access to shelter, leading to higher levels of predation from largemouth bass (*Micropterus salmoides*). Taken together, these results demonstrate that considerably more experimental work is needed to fully evaluate how the behaviour of Nile tilapia affects survivorship of native species across life stages.

### *2.4.2 Environmental modulation of behaviour*

Our study clearly showed the behaviour of Mayan cichlid was dependent on abiotic variables, including water temperature, pH and oxygen concentration. As the temperature increased, and oxygen content and pH declined, Mayan cichlid individuals were less active (Figure 2.5). By contrast, Nile tilapia increased their activity with increasing water temperature within the experimental temperature range. The contrast in their responses is suggestive of different physiological responses to elevated water temperatures, which could in turn fundamentally affect performance in competitive environments.

Several studies have quantified the thermal biology of Mayan cichlid, in part due to the potential for Mayan cichlid to be used in aquaculture (Hernández *et al.* 2014), but also because of the invasive status of Mayan cichlid in North America (Schofield *et al.* 2010). These studies have demonstrated that the species has a broad tolerance of temperatures between 15-37°C (Adams & Wolfe 2007), but performance varies across that temperature range. For example, Stauffer & Boltz (1994) suggested that the greatest survivorship of young fish was at 32.8°C in fully freshwater environments. Similarly, it has been shown that food intake and growth were highest at 33.1°C (Martinez-Palacios *et al.* 1993). By contrast the lowest levels of activity were at temperatures above 27°C, which corresponded with the lowest oxygen conditions (typically < 4 mg/L). It has long been noted that Mayan cichlid is hypoxia-tolerant (Schofield *et al.* 2009), and there is recent experimental evidence to show that the species increases ventilation rate to maintain oxygen consumption, like most oxyregulating fish species that can persist in high temperature - low oxygen habitats (Burggren *et al.* 2019). It is therefore likely that the reduced overall activity observed at higher temperatures and coincident lower oxygen conditions may also have been a behavioural response primarily to

the increasingly hypoxic conditions. Notably, it has been shown that reducing oxygen levels can reduce aggression levels in Mayan cichlids (Schofield *et al.* 2009), in contrast to the pattern seen in Nile tilapia in this study.

Experimental work on Nile tilapia has indicated that fertilization success and larval performance of the species is optimal between 24 and 32°C, above which performance declines sharply (Rana 1990; Hui *et al.* 2014). Equally, growth performance is compromised above temperatures of 32°C, with high mortality experienced by 37°C (Xie *et al.* 2011). Our experimental temperatures were between 25.6°C and 29.2°C, so within the boundaries typically tolerated by the species. It is plausible that the consistent behaviour across the thermal and oxygen gradient were linked to high hypoxia tolerance of Nile tilapia. Although the growth of the species is affected by oxygen concentration, the species is still able to grow rapidly in experimental conditions as low as 1.5 mg/L (Kolding *et al.* 2008). The contrast in performance between Nile tilapia and Mayan cichlid may be linked to different behavioural and physiological responses to the combination of warmer water and lower oxygen conditions, although comparative experiments of oxygen consumption and behaviour in standardized conditions are required.

### *2.4.3 Implications for spread of the invasive species*

The key observations of this study are that Nile tilapia is the most aggressive of the two species during the life stage studied, and that Nile tilapia is also able to maintain a high level of performance at higher temperature and lower oxygen levels than the Mayan cichlid. This is suggestive of Nile tilapia having the potential to outcompete the Mayan cichlid in more physiologically-challenging situations where shared resources are limited. The result of this study therefore parallels those of dyadic contests between invasive mosquitofish (*Gambusia*

*holbrooki*) and indigenous Iberian toothcarp (*Aphanius iberus*) in Spain (Carmona-Catot *et al.,* 2013). There, the invasive species was also the more aggressive species in the pair, and warming temperatures led to enhanced aggression and food consumption of the invader relative to the native species.

Overall, the concept that environmental variables modulate performance of species is well understood, and it is known that the distributions of species are closely matched to their physiological limits (e.g. Pörtner & Knust 2007; Payne *et al.* 2016). Building on this concept, our study showed that Nile tilapia, the species with high levels of activity across the environmental range studied, is also the species that occupies a broader geographic range in Mexico (Figure 2.1). Broad environmental tolerance is often cited as an explanation for ability of invasive species to succeed, but detailed explanations for this association are often not clear. Our study is suggestive of broad environmental tolerance allowing the invasive species to perform well during periods of environmental stress that may limit the relative performance of native species.

A final consideration is that projected climate change is likely to both raise the average temperatures of freshwater environments (e.g. Ficke *et al.* 2007), and increase the frequency of extreme events globally (Perkins *et al.,* 2012), and this may plausibly contribute to more widespread hypoxic conditions within freshwater systems. However, human activities such as use of phosphate-rich fertilizers that drive eutrophication are the main drivers of hypoxia (Jenny *et al.* 2016). Therefore, shifts in the distribution and abundance of both indigenous and invasive species may take place driven by both climate change and agricultural intensification, with those shifts mediated by physiological performance of the species. Our understanding of the effects of these future environmental changes may benefit from a more

detailed knowledge of the relative performance of species across ecological gradients, as this may strongly determine the future species composition in systems such as the freshwaters of the Yucatan peninsula.

### *Ethical considerations*

The experimental work was approved by the animal welfare and ethics review board of the University of Bristol (UIN code: UB/17/055).

# Chapter 2

# Supplementary information

**Supplementary Information 2.1: Spatial Distribution and Ecological Niche Modelling.**

Distribution data (290 records) for Nile tilapia in Mexico were obtained from GBIF on the 25 June 2019 (GBIF Occurrence Download https://doi.org/10.15468/dl.wng70o), while data for the Mayan cichlid (1390 records) were obtained on the 30 June 2019 (GBIF Occurrence Download https://doi.org/10.15468/dl.11pyhd).

To identify potential suitable habitat for Mayan cichlid and Nile tilapia in Mexico, environmental niches were modelled using Maxent 3.4.1 (Phillips *et al.* 2004). Environmental data (annual mean temperature) from the period 1960 to 1990` were downloaded at 2.5 arc minute spatial resolution from the Worldclim v.1.4 (Hijmans *et al.* 2005). Elevation data for the same scale were included, as this can represent a proxy for numerous environmental variables (Korner 2007) Prior to analyses, the downloaded distribution data were filtered to only contain unique records, resulting in 289 occurrence records for the Nile tilapia and 569 occurrences records for the Mayan cichlid. For generating models, linear, quadratic and hinge feature class options were selected to avoid model overfitting, withheld 30% of data for model testing and used a 10-fold cross validation of each model, while keeping other settings as default. A kernel density map for Mexico was created using the Kernel Density tool in ArcGIS v.10.5 (ESRI, Redlands, California). This was used by Maxent as a “bias file” to account for sampling bias when selecting background data. Model accuracy was measured using the area-under-curve (AUC) value of the receiver operating characteristic (ROC) curve, which ranges from 0.5 (no predictability) to 1 (perfect prediction), with values above 0.8 interpreted as a strong prediction.

From the Maxent model, highly suitable habitat for Nile tilapia (AUC = 0.807) was present across much of the southern region of Mexico (Figure 1a). By contrast, highly suitable habitat for Mayan cichlid was primarily identified in southeast of the country (23% of the territory), along the Atlantic slope and the coastal regions of the Yucatan Peninsula, showing a strong prediction value AUC = 0.952 (Figure 2.1b).

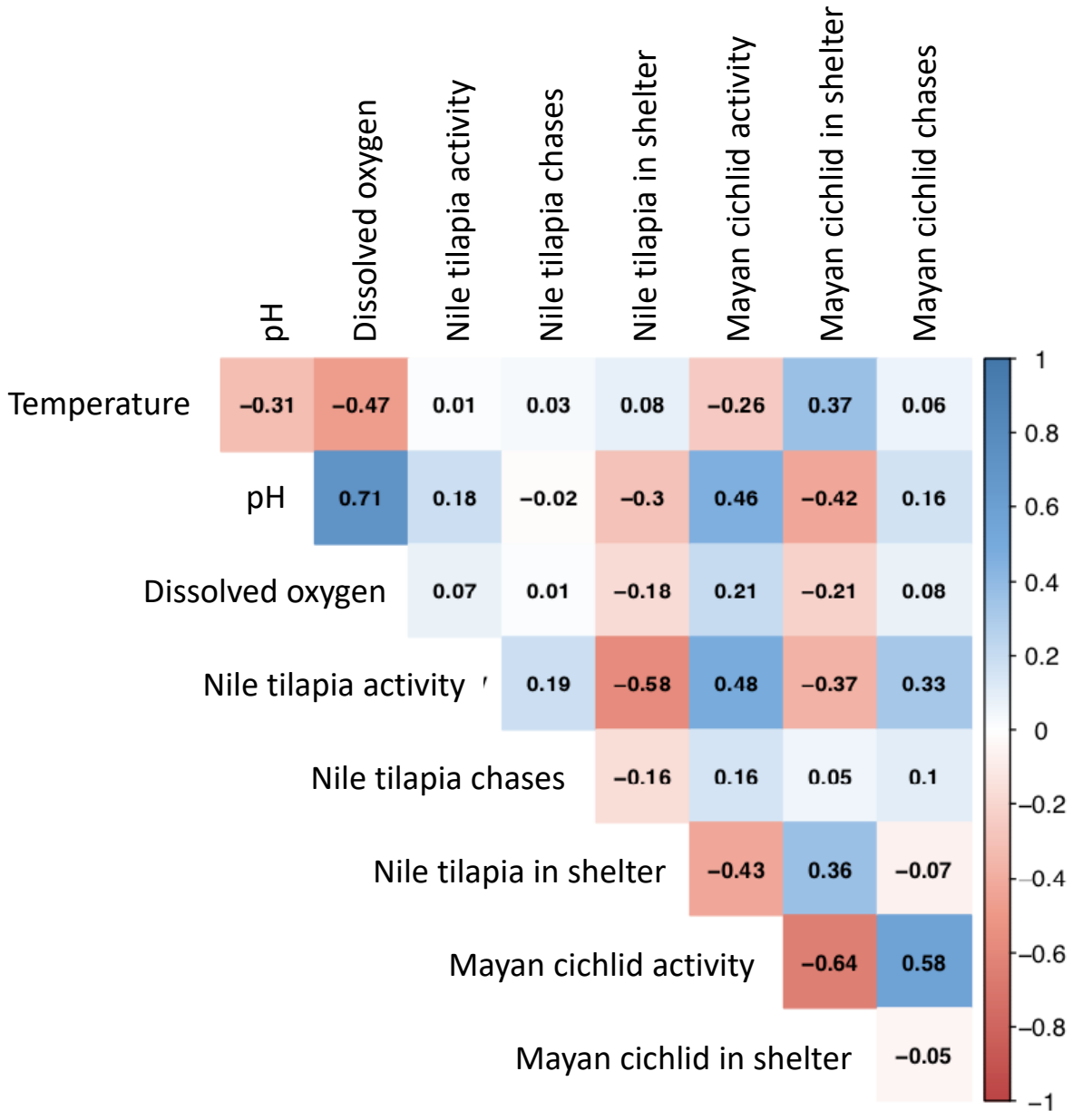
**Supplementary Information 2.2: Scoring Behaviour**

Behaviour recorded using the open source software BORIS. The column “Key” refers to the coded key that was pressed to record the behaviour observed.

| Behaviour (Point event) | Key |
|---|---|
| Nile tilapia chases Mayan cichlid | X |
| Mayan cichlid chases Nile tilapia | M |
| Nile tilapia leaves shelter area on the right | S |
| Nile tilapia leaves shelter area on the left | A |
| Mayan cichlid leaves shelter area on the right | L |
| Mayan cichlid leaves shelter area on the left | K |
| Nile tilapia returns to the shelter area | D |
| Mayan cichlid returns to the shelter area | J |

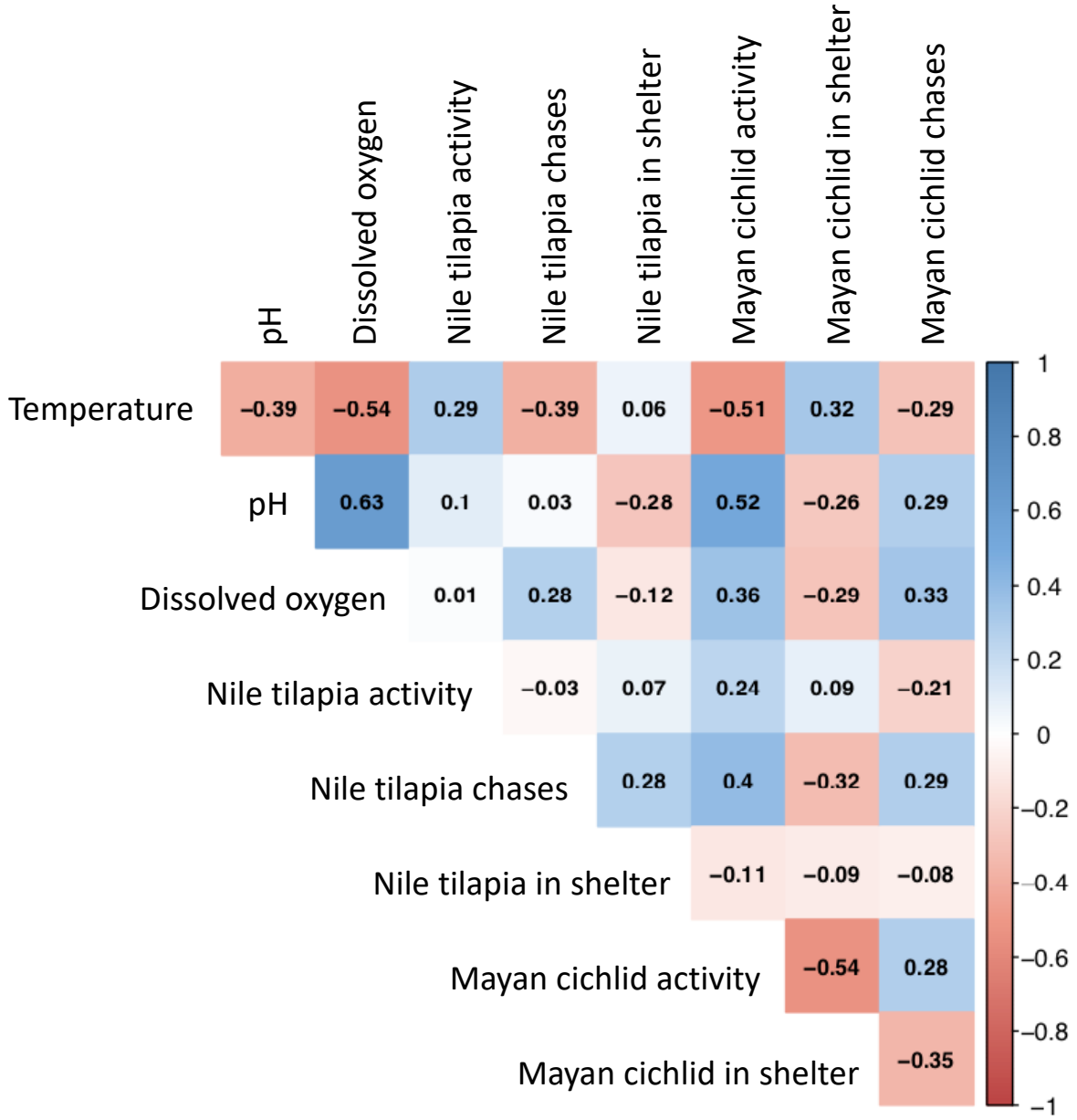
**Supplementary Information 2.3: Associations between behaviour and environment**

Pearson's correlations between behavioural and environmental variables in experiments with 5 Nile tilapia and 5 Mayan cichlids.



**Supplementary Information 2.4: Associations between behaviour and environment**

Pearson's correlations between behavioural and environmental variables in experiments with 10 Nile tilapia and 5 Mayan cichlids.

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# Chapter 3

# Quantifying the fish community of a tropical lake using environmental DNA metabarcoding.

An adapted version of this chapter is in preparation to be submitted to a peer-reviewed journal:

Gracida Juarez C.A., Collins, R.A. and Genner, M.J. Quantifying the fish community of a tropical lake using environmental DNA metabarcoding.

Author contributions: CAGJ, RAC and MJG designed the study. CAGJ conducted the field survey and eDNA extraction. CAGJ and RAC conducted the laboratory and bioinformatic analyses. CAGJ and MJG conducted the statistical analyses. CAGJ led the writing of the manuscript. MJG contributed critically to drafts.

**Abstract**

The composition in freshwater fish assemblages is frequently used as an indicator of the status of freshwater habitats. Therefore, there is growing interest in the potential for environmental DNA (eDNA) metabarcoding to provide a comprehensive evaluation of the fish species richness of freshwaters without the need for direct sampling using conventional fishing gears. While eDNA metabarcoding has reliably been shown to capture the species richness of freshwater bodies, whether eDNA can reliably be used to measure abundance and habitat use of fish species in the natural environment remains uncertain. This study compared results of eDNA metabarcoding of fish communities to results from traditional net sampling in Lake Caobas, Yucatan Peninsula, Mexico. It was found that eDNA was reliably able to identify the presence of 18 of the 20 species known from the lake, while conventional fishing yielded 14 of the species. Moreover, eDNA samples yielded a higher numbers of species per sampling event than conventional fishing, and only eDNA methods successfully identified the presence of the invasive Nile tilapia. Importantly, across all samples there was strong association between the total number of eDNA metabarcoding reads of each species and the total number of captures in conventional fishing, suggesting that eDNA data can provide a useful proxy for the relative abundance of species at the lake-wide scale. It was also found that eDNA-derived community composition data was significantly associated with water transparency and distance to shore, suggesting that eDNA has potential to capture associations between species and environmental variables. It can be concluded that eDNA-based surveys have promise for widespread use in studies of tropical freshwater assemblages, although methodological developments are required to enable eDNA metabarcoding survey approaches to be more commonly applied to freshwater conservation and management.

### 3.1 Introduction

During recent years environmental DNA (eDNA) has gained importance as a source of information on distributions of freshwater species, particularly those that are otherwise difficult to sample (Thomsen *et al.* 2016a; Valdez-Moreno, *et al.* 2019). Environmental DNA has been of specific use for monitoring the distribution of species of conservation concern (Thomsen *et al.* 2012b; Stoeckle *et al.* 2016), and surveying habitats for the presence of invasive species (Klymus *et al.* 2017; Qu *et al.* 2019, Valdez-Moreno *et al.* 2019). Due to an enhanced understanding of sampling and analytical protocols, the reliability on this tool is improving. Advances in eDNA metabarcoding methods and associated bioinformatics now enable resolution of the presence of large number of species from a relatively small number of eDNA samples, and there are strong indications that eDNA metabarcoding can outperform conventional capture-based methods at species detection in freshwater systems (Ji *et al.* 2013).

Despite an improved understanding of methods to capture and process eDNA samples, there are considerable gaps in our knowledge, particularly about the use of metabarcoding methods for inferring the abundance of species in communities, and habitat use patterns within the environment. These are important because management and conservation applications, as well as ecologists undertaking fundamental research, often require detailed knowledge of the abundance and local distributions of species in an environment. There are several well-reported issues that determine the utility of eDNA for accurately quantifying the community. Of central consideration is the persistence of eDNA and the rates transport of the eDNA from the source, which determine the likelihood that eDNA results accurately representing a detection of the species in the sampled environment. Moreover, eDNA may

be more abundant for some species at certain times of the year, for example during breeding periods, or at times of high mortality. There are also concerns that in metabarcoding applications the universal primers typically employed may favour some species while others fail to amplify. This would decouple any association between metabarcode read counts assigned to a species and the actual abundance of that species in the environment. Such issues may explain the inconsistent associations between eDNA abundance and species abundance sometimes reported (e.g. Hinlo *et al.* 2017).

Nevertheless, despite concerns about how reliably eDNA may reflect abundance and space use in freshwater environments, there are indications that eDNA results may be useful for providing more detailed information than presence-absence alone. For example, across water bodies, eDNA abundance of lake trout (*Salvelinus namaycush*) correlate significantly with capture-based sampling estimates (Lacoursière-Roussel *et al.* 2015), suggesting eDNA reads can be used as a proxy for absolute abundance. Even within still water bodies, eDNA-based metabarcode read counts have shown to decrease significantly with increasing distance from the source organism over a spatial scale of less than 100 m (Li *et al.* 2019), suggesting they may have capacity to resolve abundance over extremely fine spatial scales. Moreover, even in a more dynamic riverine environment, the abundance of eDNA reads associates strongly with the number of migrating coho salmon (*Oncorhynchus kisutch*) and sockeye salmon (*Oncorhynchus nerka*), after correcting for river flow rate (Levi *et al.* 2019), indicating capacity for eDNA to provide good temporal resolution of changing abundance. Evidence from multispecies eDNA metabarcoding studies also suggests that in whole community comparisons, eDNA read counts can correlate broadly positively with abundance estimates from conventional capture-based field sampling (Hanfling *et al.* 2016), similar to results from

marine systems (Thomsen *et al.* 2016). Importantly, however, there are still very few studies that have directly compared read counts from eDNA metabarcoding with results from capture-based conventional sampling in freshwater systems.

This study aims to evaluate the application of eDNA for assessment in tropical freshwaters, focussing on Lake Caobas in the Yucatan Peninsula, in Mexico. This region of Mexico has a rich freshwater fish fauna (Contreras *et al.* 2014), but eDNA-based analyses have been limited to Lake Bacalar and the neighbouring Rio Hondo (Valdez-Moreno *et al.* 2019). That study, based on COI showed that fish species could be detected using eDNA from water and sediment samples, although no comparisons were made with census data collected using conventional capture-based survey methods. Therefore, this study aims to build on this knowledge by a) evaluating the ability of fish-specific 12S primers to recover the full species of the tropical lake Caobas and determining the optimal sampling effort required to capture the species richness, b) testing for associations between eDNA read abundance and fish abundance in a capture-based survey looking for the reliability of eDNA to resolve the composition of native fish community, which could inform its use for sampling other tropical lakes in the region, and c) evaluating if fish spatial community structure associates with measured environmental variables, including water depth, temperature, oxygen concentration and distance from the shore. The interpretation of results is focussed on the potential benefits and limitations of eDNA-based monitoring in tropical freshwater lakes.

## 3.2 Methods

### *3.2.1 Sampling sites*

Lake Caobas is located in the south of the state of Quintana Roo (approximately 18.450°N, 89.098°W) in the Yucatan Peninsula, Mexico (Figure 3.1). The lake is at 100 m altitude, has a surface area of approximately 0.2 $km^2$, a maximum depth of approximately 2.5 m, a mean depth of approximately 2 m, and is mainly recharged by an underground water source. The lake is surrounded by a mosaic of forest (primarily to the west and south), shrubs and herbaceous vegetation (primarily to east) and semi-intensive cultivation (primarily to the north-west). The lake is very close to the town of Caobas, and inhabitants use the lake for small-scale fishing and pumping water for cattle. In the semi-intensive cultivation areas cattle commonly use the lake margins for drinking, causing erosion and introducing manure.

Sampling took place on the 23rd and 24th of July 2018. Both eDNA sampling and capture fishing (gill netting) took place at 16 sampling sites distributed across the lake. Eight sites were sampled the first day between the 12:30 and 19:00 hours, and eight sites were sampled the second day between the 07:30 and 13:30 hours (Figure 3.1, Table 3.1). The location of each sample was recorded with a handheld geographic positioning system (GPS) (Garmin, Lenexa, Kansas, USA), and several environmental variables were measured. Surface water temperature and pH were measured using a portable pH-meter (ExStik, China), surface dissolved oxygen measured using a portable dissolved oxygen meter (HI-9146, Hanna Instruments, Romania). Depth and water transparency were measured with a secchi disc (32 cm diameter

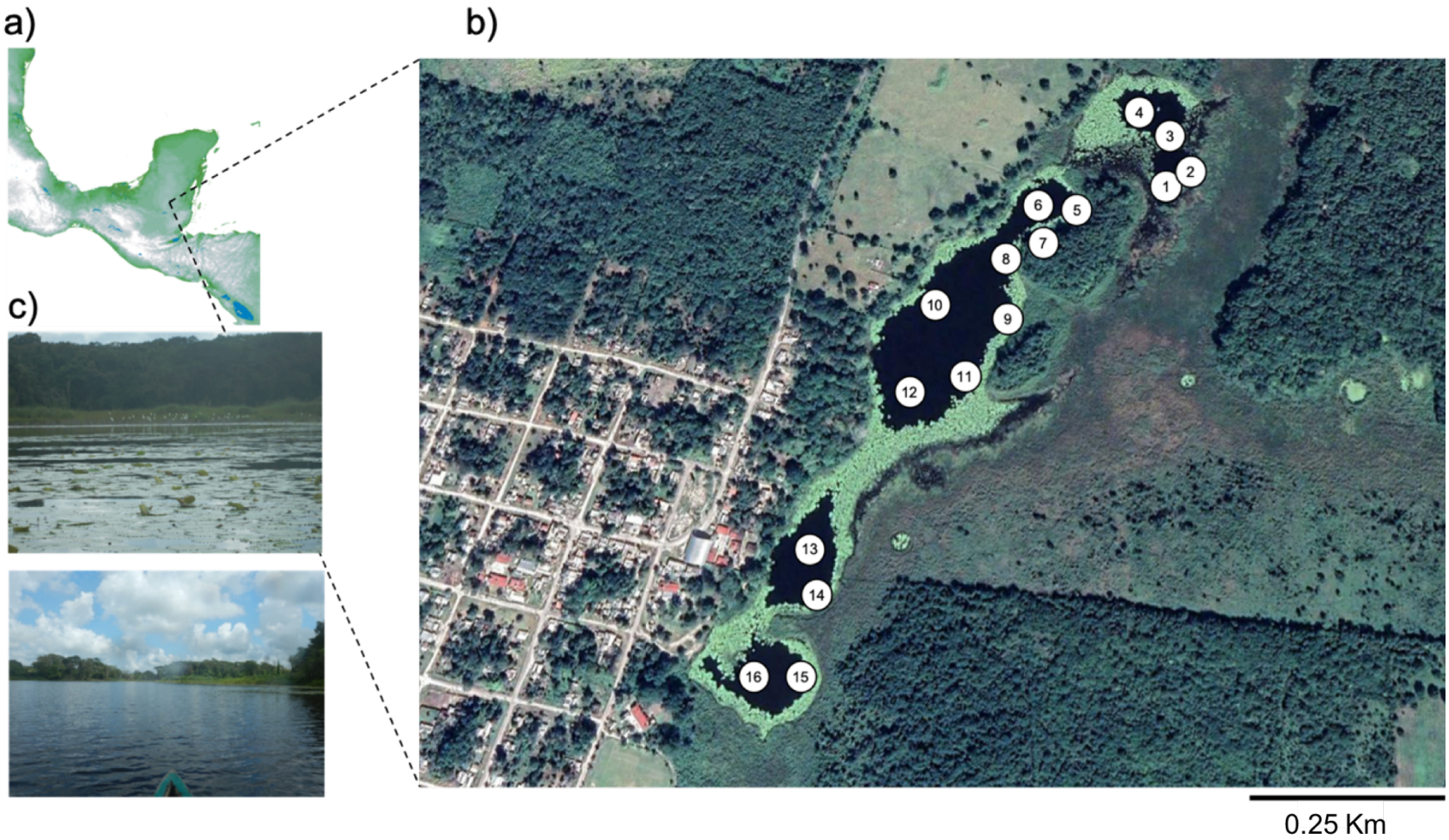


**Figure 3.1.** Location of Lake Caobas on a) Yucatan Peninsula in Mexico; b) Circled numbers show location of the sampling sites; c) images showing the nearshore macrophytes and open waters that characterise the lake.

**Table 3.1**. Environmental parameters at each of the 16 sites on Lake Caobas, sampled 23 and 24 July 2018.

| Site Code | Longitude °N | Latitude °W | Sampling date | Temperature (°C) | pH | Dissolved oxygen (mg/L) | DO % Saturation | Transparency (cm) | Depth (cm) | Distance to the shore (m) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 18.4518 | -89.0956 | 23-07-2018 | 32.6 | 8.00 | 7.09 | 97.66 | 38 | 95 | 26.83 |
| 2 | 18.45205 | -89.0954 | 23-07-2018 | 32.9 | 8.11 | 7.26 | 102.22 | 40 | 150 | 32.91 |
| 3 | 18.45256 | -89.0957 | 23-07-2018 | 33.5 | 8.10 | 6.81 | 96.84 | 20 | 158 | 81.85 |
| 4 | 18.45283 | -89.0961 | 23-07-2018 | 33.3 | 8.07 | 6.96 | 98.65 | 120 | 120 | 34.70 |
| 5 | 18.45148 | -89.0969 | 23-07-2018 | 33.6 | 8.10 | 6.34 | 90.31 | 180 | 180 | 8.19 |
| 6 | 18.45144 | -89.0972 | 23-07-2018 | 33.4 | 8.16 | 6.10 | 87.88 | 140 | 230 | 29.31 |
| 7 | 18.45111 | -89.0973 | 23-07-2018 | 32.8 | 8.24 | 6.14 | 86.3 | 145 | 250 | 33.29 |
| 8 | 18.45082 | -89.0978 | 23-07-2018 | 33.0 | 8.23 | 5.95 | 83.91 | 150 | 210 | 14.03 |
| 9 | 18.44998 | -89.0978 | 24-07-2018 | 29.3 | 8.14 | 5.23 | 69.24 | 100 | 240 | 34.81 |
| 10 | 18.45026 | -89.0986 | 24-07-2018 | 30.3 | 8.09 | 5.18 | 69.92 | 120 | 200 | 20.33 |
| 11 | 18.44889 | -89.0983 | 24-07-2018 | 30.4 | 8.16 | 5.76 | 77.73 | 130 | 230 | 43.40 |
| 12 | 18.44899 | -89.0992 | 24-07-2018 | 30.5 | 8.12 | 5.58 | 75.43 | 130 | 250 | 42.22 |
| 13 | 18.44669 | -89.1004 | 24-07-2018 | 31.2 | 7.96 | 4.95 | 67.72 | 130 | 250 | 16.44 |
| 14 | 18.44592 | -89.1004 | 24-07-2018 | 31.1 | 7.97 | 4.81 | 65.69 | 160 | 250 | 17.40 |
| 15 | 18.44491 | -89.1003 | 24-07-2018 | 31.5 | 7.99 | 5.05 | 69.44 | 120 | 250 | 35.41 |
| 16 | 18.44498 | -89.1012 | 24-07-2018 | 31.8 | 8 | 5.00 | 69.11 | 110 | 220 | 20.46 |
| Average values | | | | 31.95 | 8.09 | 5.89 | 81.75 | 114 | 205 | 30.72 |

### *3.2.2 eDNA sampling*

Two replicate water samples were collected from the surface of each sampling site using sterilized PET bottles of 600 ml capacity, wearing latex gloves that were disposed of after each sampling event. Sample bottles were placed into a cooler before being filtered in the field using sterile 60 ml syringes connected to Sterivex filter unit (0.22 µm pore size; Millipore, Merck KGaA, Darmstadt, Germany). One filter unit was used for contents of each bottle, and 100% ethanol was used to preserve the contents. The filter unit was then placed inside a sterile sealable bag, stored initially at -5 °C before being transferred to -20°C for long-term storage. During the filtering process, new latex gloves were worn when handling each water sample to avoid cross-contamination.

### *3.2.3. Conventional net sampling*

A gill net was placed at each sample site for 1 hour. The net was a CEN standard multimesh monofilament, measuring 30 m long x with a stretched depth of 1.5 m, and twelve panels each 2.5 m long with the following mesh sizes: 43 mm, 19.5 mm, 6.25 mm, 10 mm, 55 mm, 8 mm, 12.5 mm, 24 mm, 15.5 mm, 5 mm, 35 mm, and 29 mm. Specimens captured were individually identified, labelled and photographed, and then preserved in 100% ethanol. Specimens have been deposited in the ichthyological collection of El Colegio de la Frontera Sur, Unidad Chetumal, in Chetumal, Quintana Roo, Mexico. Those specimens to be used in generation of the 12S barcode reference library (see below) had a pectoral fin clip taken and preserved in 100% ethanol, prior to preservation of the whole fish.

### *3.2.4 eDNA extraction*

In a dedicated clean eDNA extraction laboratory, and using sterilized equipment, the plastic cover of the filter unit was detached, and the filter paper removed. The filter paper of each sample was placed into a 1.5 ml microcentrifuge tube and DNA extracted using a DNeasy Blood & Tissue Kit (Hilden, Germany). Extracted DNA samples were stored in dedicated freezer at -20°C. For a complete description of the DNA extraction procedure see Supporting Information.

### *3.2.5 DNA amplification, samples pooling and 12S Library preparation*

The procedure in this part of the survey was to use the 1-step PCR protocol for amplifying the MiFish 12S fragment (Miya *et al.* 2015), using the primers (MiFish-U-F, 5'-GCC GGT AAA ACT CGT GCC AGC-3'; MiFish-U-R, 5'-CAT AGT GGG GTA TCT AAT CCC AGT TTG-3'). These primers were chosen because they target a hypervariable region of the 12S fragment that contains sufficient information to reliably identify fishes to taxonomic family, genus and species, while being sufficiently versatility to amplify the fragment in a diverse range of fish species (Miya *et al*. 2015, Yamamoto *et al*. 2017). The metabarcoding primers each had an 8-base sample-tag attached, and each tag had at least three differences from the eight bases from other tags (Supplementary Information 3.1). Primers also had a variable number (2–4) of leading Ns, in order to increase sequence variability to improve Illumina sequencing (Supplementary Information 3.1). Each forward and reverse primer had the same sample-tag attached to both ends, and in total there were 96 different primer pairs enabling up to 96 samples to be multiplexed in each library.

Each individual PCR reaction used the following reagents: 10µl AmpliTaq Gold Master Mix; 0.16 µl BSA; 5.84 µl RNAase-free $H_20$; 2 µl forward and reverse primer mix 5 µM; 2 µl DNA Template. Each reaction was performed by triplicate, each plate contained 3 negative controls including RNAase-free $H_20$ as a substitute of the template and mixed with the reagents in the same proportions; and 11 empty wells denominated as blanks. The PCR programme consisted of a denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. There was then a final extension step of 72°C for 5 minutes. To evaluate amplification success, 2 µl of each PCR product was run on agarose gel.

To generate the final library, 18 µl each sample PCR product, including the negatives, were pooled together in a single Eppendorf and homogenized by vortexing. The pooled samples were purified using a MinElute column (Qiagen, Hilden, Germany) to remove DNA fragments shorter than 70 bp, before concentrating the DNA approximately ten times. The DNA concentration was measured using a Qubit fluorimeter (Life Technologies, Carlsbad, CA, USA) with the Broad-Range DNA quantification kit, yielding a final concentration of 75 ng/µl. For library preparation, a PCR-free ligation protocol with the NEXTflex PCR-Free DNA sequencing kit (BIOO Scientific, Austin, TX) was used, following the manufacturer's instructions. The concentration of the library was calculated using a NEBNext Library Quant kit (New England Biolabs, Hitchin, UK) against the kit standards using an Eco48 real-time PCR system (PCRmax Limited, Stone, Staffordshire, UK). The final library (18 pM) was sequenced on an Illumina NextSeq 500 platform at the University of Bristol genomics facility, using the v2.5 x 150 bp mid-output kit, including a 1% PhiX control.

### *3.2.6 12S reference library of fish species present in Lake Caobas*

Preserved fin tissue was available for 17 species (Table 3.2). DNA was extracted using the Wizard extraction protocol (Promega, Fitchburg, WI, USA), and samples normalised to ~50 ng/µL. A ~612 bp amplicon of 12S/tRNA-Phe (567 bp without primers) was amplified encompassing the MiFish metabarcode fragment (Miya *et al.* 2015), using the forward primer Aa22-12SF 5'-AGC ATA ACA CTG AAG ATR YTA RGA-3', and the reverse primer Aa633-12SR 5'-TTC TAG AAC AGG CTC CTC TAG-3'. PCR was conducted in 20 µL reactions using: 10 µL GoTaq Green Master Mix (Promega, Madison, USA), 2ul forward primer (2 mM), 2 µl reverse primer (2mM), 1 µL of template DNA and 5µL of water. PCR involved an initial denaturation of 2 minutes at 95°C. This was followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 45 seconds. Finally, there was a final extension step of 72°C for 5 minutes. PCR products were cleaned using a Zymo-Spin IC column (Zymo Research Corporation, Tustin, CA), and sequenced by Eurofins Genomics using ABI technology.

**Table 3.2.** Fish species present in Lake Caobas, including the number of reads obtained through eDNA metabarcoding, the number of specimens captured between the 23 and 24 of July of 2018, using gill nets, and the number of individuals sequenced for the 12S reference library.

| Species | Family | Code | Common name | Total number of metabarcode reads | Total individuals captured in survey | Tissue samples sequenced for reference library |
|---|---|---|---|---|---|---|
| *Astyanax bacalarensis* | Characidae | Aba | Bacalar tetra | 3,539,933 | 145 | 2 |
| *Atherinella alvarezi* | Atherinopsidae | Aal | Gulf silverside | 247,522 | 15 | 2 |
| *Belonesox belizanus* | Poecillidae | Bbe | pike topminnow | 72,932 | 2 | 2 |
| *Cribroheros robertsoni* | Cichlidae | Cro | false firemouth cichlid | 315,741 | 9 | 1 |
| *Cryptoheros chetumalensis* | Cichlidae | Cch | red fin spilurus cichlid | 0 | 0 | 2 |
| *Dorosoma petenense* | Clupeidae | Dpe | threadfin shad | 5,452,008 | 209 | 2 |
| *Gambusia sexradiata* | Poecillidae | Gse | teardrop mosquitofish | 81 | 0 | 1 |
| *Gambusia yucatana* | Poecillidae | Gyu | Yucatan gambusia | 95 | 0 | 1 |
| *Hyphessobrycon compressus* | Characidae | Hco | Mayan tetra | 4,122 | 2 | 2 |
| *Mayaheros urophthalmus* | Cichlidae | Mur | Mayan cichlid | 9,900 | 7 | 2 |
| *Oreochromis* sp. | Cichlidae | Ore | tilapia | 304,572 | 0 | 3 |
| *Parachromis friedrichsthalii* | Cichlidae | Pfr | yellowjacket cichlid | 51,861 | 2 | 1 |
| *Petenia splendida* | Cichlidae | Psp | bay snook | 16,477,646 | 7 | 2 |
| *Poecilia kykesis* | Poecillidae | Pky | Péten molly | 0 | 1 | - |
| *Poecilia mexicana* | Poecillidae | Pme | shortfin molly | 170,531 | 8 | - |
| *Rhamdia guatemalensis* | Heptapteridae | Rgu | pale catfish | 363 | 6 | 2 |
| *Rocio octofasciata* | Cichlidae | Roc | Jack Dempsey | 3,937 | 0 | - |
| *Thorichthys meeki* | Cichlidae | Tme | firemouth cichlid | 1,774,466 | 27 | 2 |
| *Trichromis salvini* | Cichlidae | Tsa | yellow belly cichlid | 7,638 | 0 | 2 |
| *Vieja melanura* | Cichlidae | Vme | redhead cichlid | 1,170,724 | 5 | 4 |

### *3.2.7 Metabarcoding bioinformatics.*

The Illumina raw sequencing data were converted to fastq format using bcl2fastq v2.20 (Illumina Inc, Great Abington, Cambridge). The fastq files were then cleaned, demultiplexed, and OTUs obtained using the scripts from the crack-pipe pipeline (https://github.com/boopsboops/crack-pipe), see Supplementary Information. Briefly, paired-end reads were merged, reads orientated to 5' to 3' direction, and low-quality reads were removed. Next, the reads files were demultiplexed using the sample barcodes, and PCR primers and barcodes removed. Reads were then quality filtered and dereplicated, providing the number of unique sequences present on a per-sample basis. Next, reads from samples were merged, dereplicated again globally, and sequences clustered into groups. Finally, an output quality filter was applied that discarded spurious low abundance cluster groups, before an OTU table was generated for remaining cluster groups from the reference library. The table was then simplified to represent only the number of reads per species per sampling site.

### *3.2.8 Data analyses*

To determine the optimal sampling effort needed to capture the species richness of the community using either eDNA metabarcoding or fish capture data, sample-based rarefaction was performed using the "specaccum" function in the package "Vegan" (Oksanen *et al.* 2019) in R 3.6.2 (R Core Team 2019). Associations between the total number of reads of a species [$\log_{10}(x+1)$ transformed] and the number of fish captured both were quantified using linear models in base R. The packages "ggplot" (Wickham, 2016) and "ggpubr" (Kassambara, 2019) were used to plot the confidence intervals of models.

To ordinate associations between fish community composition (measured using presence-absence from eDNA metabarcoding or fish capture data), the metaMDS and envfit functions from "Vegan" were used. These MDS analyses included all recorded species, alongside the six measured environmental variables (water temperature, depth, transparency, pH, oxygen and distance to shore). The statistical significance of the effects of individual variables was tested using a permutational multivariate analysis of variance using distance matrices, implemented with the adonis2 function in "Vegan", with default settings.

## 3.3 Results

### *3.3.1 Species detection using eDNA and gill net surveys*

The samples from Lake Caobas were run alongside samples for one other project. The total number of raw reads for both projects present in the fastq files obtained from Illumina sequencer was 83,773,428. After merging, re-orientating and removing low quality reads, the resultant number of sequences was 82,042,557. Then, demultiplexing the reads by sample barcodes left 79,133,815 reads, and after trimming the sequencing adapters 79,061,384 reads remained. Then, after further filtering by quality 55,160,868 sequences were left.

Across the 32 samples from Lake Caobas, there were 29,863,074 reads assigned to 17 native species and one invasive taxon (*Oreochromis* sp.) (Table 3.2). The most common species in the reads were *Petenia splendida*, *Dorosoma petenense* and *Astyanax bacalarensis*. Notably, the eDNA only failed to detect two species that have been reported from the lake, namely *Criptoheros chetumalensis* and *Poecilia kykesis.* The average number of species caught per sampling site was 11.37 (95% CI ± 1.60). The species accumulation curve indicated that is

possible to record most species present in the eDNA samples by sampling 9 of the 16 sites (Figure 3.2a).

Across the 16 gill net sets from Lake Caobas, a total number of 445 specimens were captured belonging to 14 species. The most common species were *Dorosoma petenense* (209 specimens), *Astyanax bacalarensis* (145 specimens), and *Thorichthys meeki* (27 specimens). The net samples failed to detect seven species known from the lake, namely *Trichromis salvini*, *Cryptoheros chetumalensis*, *Paraneetroplus synspilum Gambusia sexradiata*, *Gambusia yucatana*, *Rocio octofasciata*, and the invasive *Oreochromis* sp. The average number of species detected per sampling site was 4.5 (95% CI ± 0.93). The species accumulation curve demonstrated that using 13 gill net sets are enough to record most species present in this lake (Figure 3.2b).

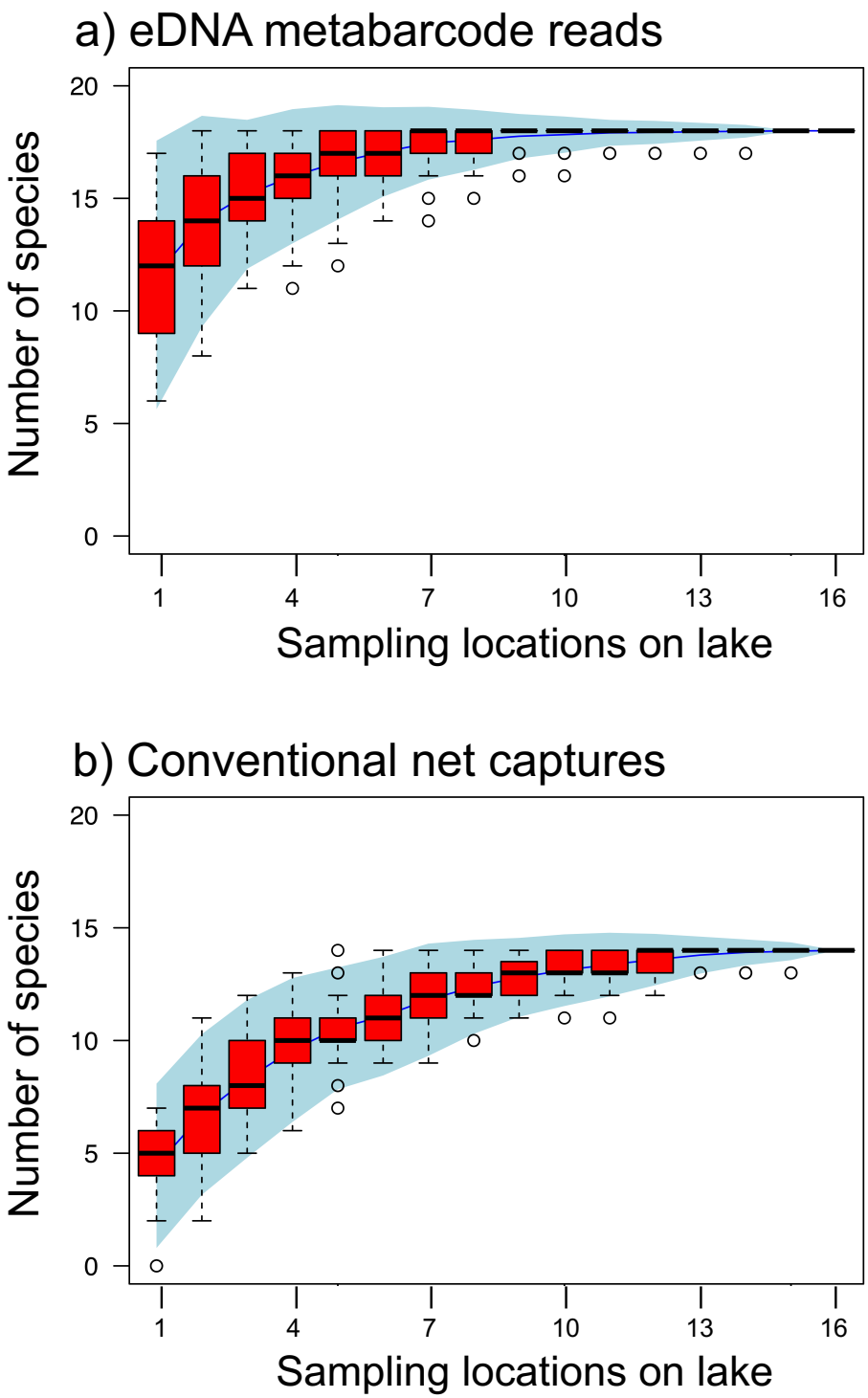


**Figure 3.2.** Species accumulation curves for a) eDNA metabarcoding reads and b) conventional net survey captures at Lake Caobas. Red boxplots indicate median, upper and lower quartiles. Blue background indicates the 95% confidence intervals of the mean.

### *3.3.2 Associations between the number of eDNA reads and specimens captured*

In cross-species comparisons across all sampling events, there was a strong positive association between the total number of sequences assigned to a species and the total number of individuals captured [$r^2$ = 0.40, $P$ = 0.003; both variables $\log_{10}(x+1)$ transformed] (Figure 3.3). In cross-species comparisons within the sampling sites where captures were made, 14 of the 15 associations were positive. Five of the 15 comparisons were statistically significant ($P < 0.05$; Figure 3.4; Table 3.3). When comparing within species, no significant associations between the number of reads at a site, and the total captures at the site [$\log_{10}(x+1)$ transformed] were found, for the four most abundant species (Table 3.4).

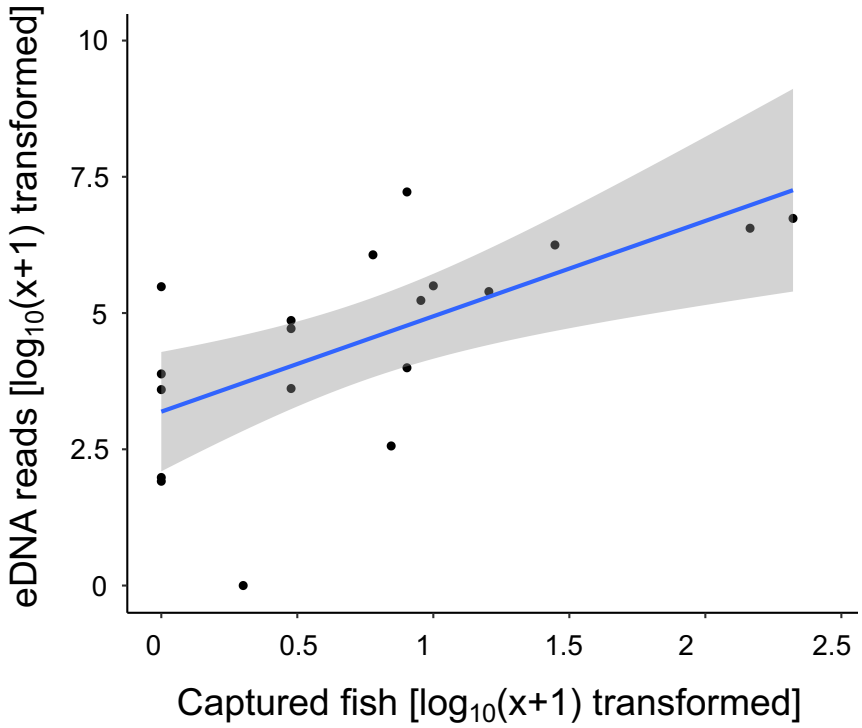


**Figure 3.3.** Association between the total number of reads and the total number of individual fish captured across all sampling locations in Lake Caobas. Each filled circle represents one species. Shaded area indicates the 95% confidence interval. $r^2$ = 0.40, $F_{1,19}$ = 11.520, $P$ = 0.003.

**Table 3.3.** Associations between the number of metabarcoding reads [response variable, $\log_{10}(x+1)$ transformed) and specimens captured [predictor variable, $\log_{10}(x+1)$ transformed), for the sampling sites in Lake Caobas. **Bold** indicates statistically significant results.

| Site Code | Slope | $r^2$ | $F_{1,17}$ | $p$ |
|---|---|---|---|---|
| 1 | 1.977 | 0.23 | 5.079 | **0.037** |
| 2 | 4.370 | 0.35 | 9.153 | **0.008** |
| 3 | 3.031 | 0.19 | 3.977 | 0.062 |
| 4 | 1.968 | 0.17 | 3.546 | 0.077 |
| 5 | 2.626 | 0.04 | 0.705 | 0.413 |
| 6 | 5.208 | 0.50 | 16.990 | **< 0.001** |
| 7 | -0.349 | 0.01 | 0.162 | 0.693 |
| 8 | 0.437 | 0.02 | 0.333 | 0.571 |
| 9 | 4.398 | 0.25 | 5.623 | **0.030** |
| 10 | 2.075 | 0.17 | 3.604 | 0.075 |
| 11 | 0.707 | 0.01 | 0.222 | 0.644 |
| 12 | 2.317 | 0.07 | 1.361 | 0.259 |
| 13 | 2.656 | 0.17 | 3.461 | 0.080 |
| 14 | 2.355 | 0.11 | 2.111 | 0.165 |
| 15 | - | - | - | - |
| 16 | 3.091 | 0.32 | 7.817 | **0.012** |
| All combined | 1.750 | 0.40 | 11.520 | **0.003** |

- No fish captured in conventional sampling at site 15.

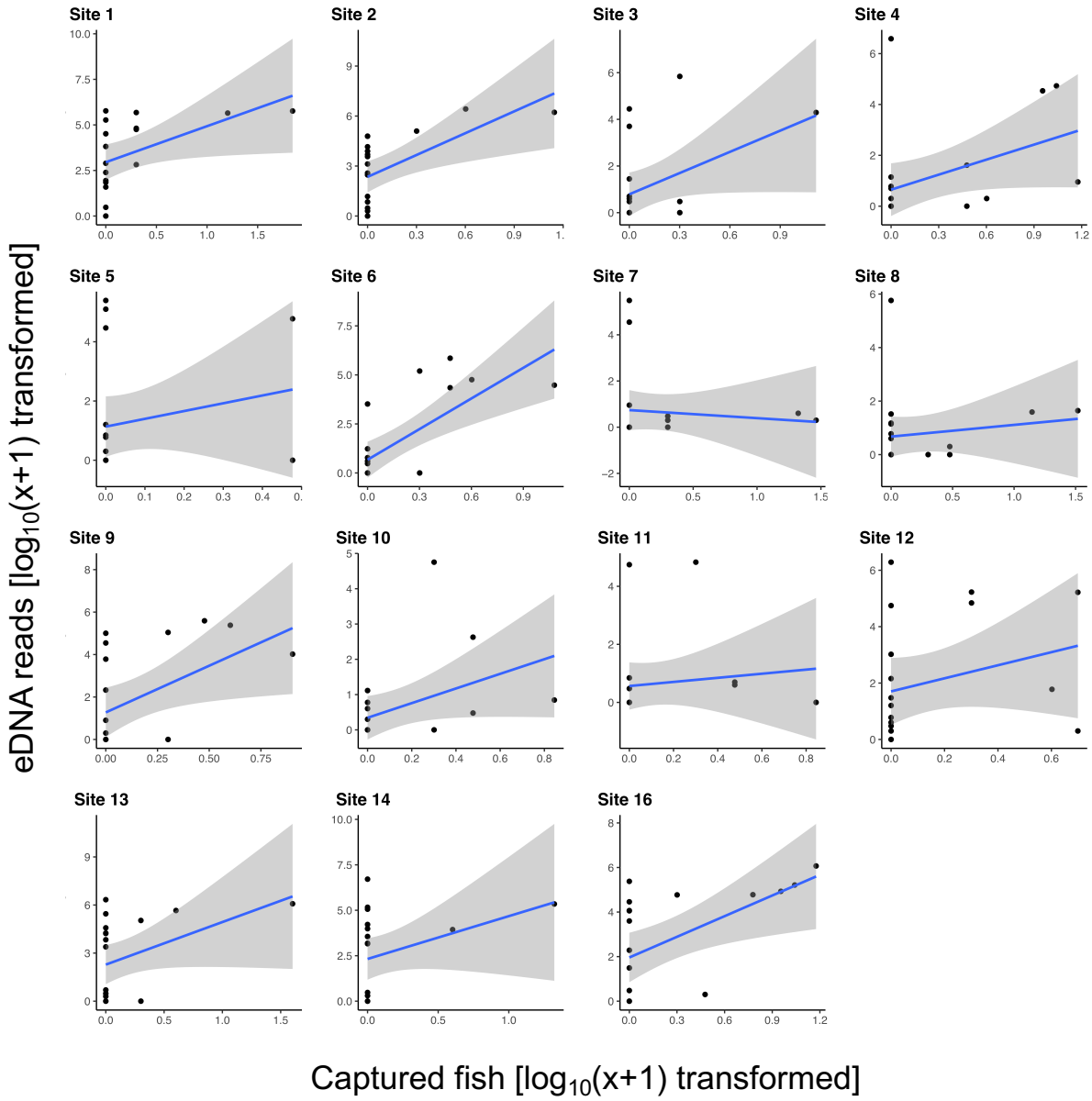


**Figure 3.4.** Associations between the total number of reads and the total number of individual fish captured at each sampling locations in Lake Caobas. Each filled circle represents one species. Note no fish were captured at Site 15.

**Table 3.4**. Associations between the number of reads and specimens captured for the four most common species within eDNA reads across the sampled sites in Lake Caobas. * Total reads, **Rarefied reads.

| Species | N sampling locations specimens captured | N sampling locations reads recorded | Correlation between reads and captures across sites (Spearman's r) * | *P* | Correlation between reads and captures across sites (Spearman's r) ** | *P* |
|---|---|---|---|---|---|---|
| *Astyanax bacalarensis* | 12 | 16 | -0.016 | 0.951 | 0.000 | 1.000 |
| *Atherinella alvarezi* | 3 | 9 | 0.145 | 0.593 | 0.161 | 0.550 |
| *Dorosoma petenense* | 14 | 15 | -0.112 | 0.678 | -0.295 | 0.266 |
| *Thorichthys meeki* | 11 | 16 | -0.027 | 0.919 | 0.088 | 0.745 |

### *3.3.3 Associations between community structure and environmental variables.*

Non-metric multi-dimensional scaling plots characterized the presence-absence of species in communities resolved using both eDNA and net captures (Fig. 3.5). While the analysis identified species commonly recovered together in both datasets, there were no clear clusters of sites, or combinations of variables that consistently and reliably predicted community structure in both datasets. Only pH showed a significant association with eDNA-resolved community structure, with most species showing an overall negative association with increasing pH (Table 3.5; Fig.3.5). No environmental variables were reliable predictors of community structure as resolved using the net samples (Table 3.5).

**Table 3.5.** Quantification of associations between community similarity and environmental variables, using permutational multivariate analysis of variance using distance matrices.

| Response variable | Predictor | Df | SS | r2 | F | P |
|---|---|---|---|---|---|---|
| eDNA community (presence-absence) | depth | 1 | 0.030 | 0.059 | 1.036 | 0.423 |
| | transparency | 1 | 0.012 | 0.023 | 0.412 | 0.754 |
| | shore | 1 | 0.049 | 0.096 | 1.701 | 0.194 |
| | pH | 1 | 0.139 | 0.270 | 4.769 | **0.013** |
| | oxygen | 1 | 0.013 | 0.025 | 0.447 | 0.718 |
| | temperature | 1 | 0.009 | 0.017 | 0.301 | 0.815 |
| | Residual | 9 | 0.261 | 0.509 | | |
| | Total | 15 | 0.513 | 1.000 | | |
| net captures community (presence-absence) | depth | 1 | 0.156 | 0.080 | 1.157 | 0.374 |
| | transparency | 1 | 0.084 | 0.043 | 0.625 | 0.682 |
| | shore | 1 | 0.180 | 0.093 | 1.341 | 0.298 |
| | pH | 1 | 0.143 | 0.074 | 1.065 | 0.433 |
| | oxygen | 1 | 0.070 | 0.036 | 0.524 | 0.744 |
| | temperature | 1 | 0.236 | 0.121 | 1.755 | 0.163 |
| | Residual | 8 | 1.076 | 0.553 | | |
| | Total | 14 | 1.947 | 1.000 | | |

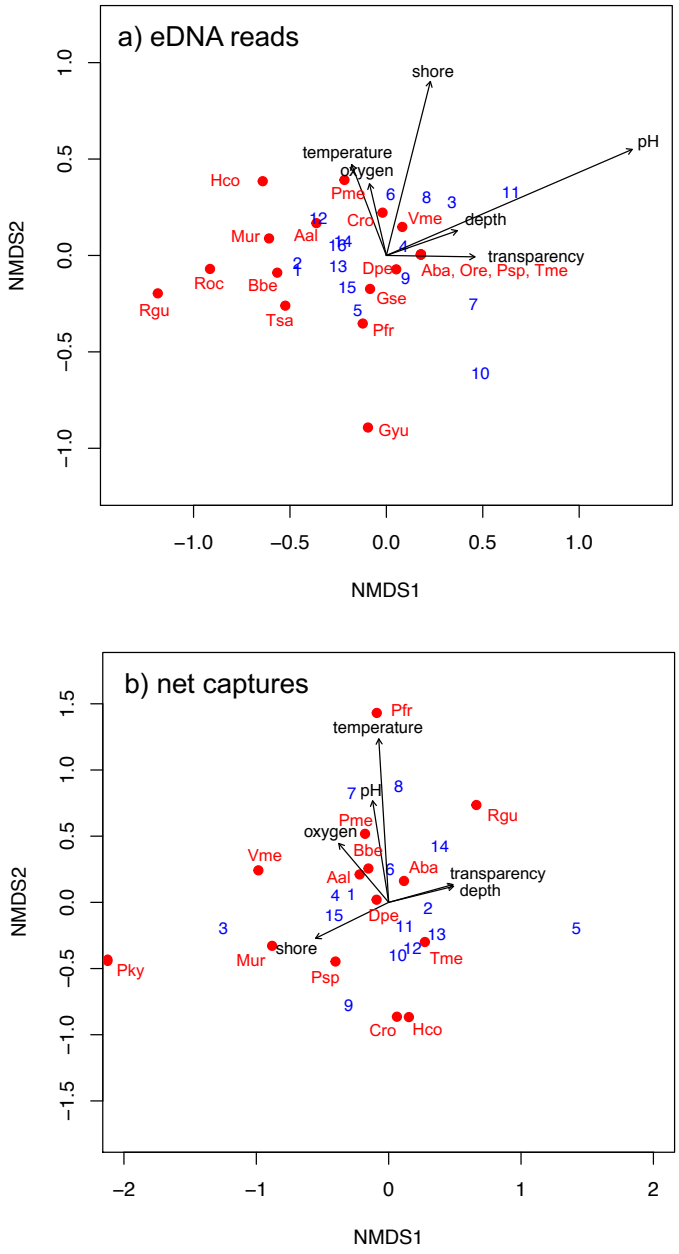


**Figure 3.5.** Non-metric multidimensional scaling plots illustrating community similarity (presence-absence) at sampling sites (blue) in Lake Caobas. Closer points indicate more similar communities. Species are overlaid in red (see Table 3.2 for codes), and environmental variables are shown in black.

### 3.4 Discussion

Here, an intensive study of the small tropical Lake Caobas was undertaken to evaluate the prospects of eDNA analyses to inform research and management of tropical freshwaters. The lake is known to contain 20 species of fish, and 18 of them were recovered using eDNA barcoding (32 samples across 16 sites), and 14 of them using multi-mesh gill net captures (16 hours of fishing, across 16 sites). Notably, there were some differences in the species sampled using the different methods. Only eDNA was able to sample the small-bodied *Gambusia sexradiata and Gambusia yucatanica,* that are too small to be reliably caught in standard survey gill nets. Similarly, only eDNA was able to sample *Trichromis salvini*, *Rocio octofaciatus* and *Oreochromis niloticus*. This may reflect these species preferring to occupy habitat that was not sampled directly, for example shallow water macrophyte beds, or it may be a consequence of net avoidance behaviour of the species. By contrast, only the gillnet successfully sampled *Poecilia kykesis*, but this species was absent from 12S reference library and therefore it remains unclear if it eDNA reads belonging to this species were present. Only one species was not detected by either method, *Cryptoheros chetumalensis*, and this species was present in the 12S reference library. The absence of this species may reflect the rarity of the species in the lake, use of a specific (and unsampled) microhabitat by the species, and/or low rates of DNA release. The results confirm that eDNA-based methods can at the very least be viewed as a complementary to capture-based survey methods, capable of detecting species that are difficult to survey otherwise (Thomsen *et al.* 2012; Shaw *et al.* 2016; Kelly *et al.* 2017).

### *3.4.1 Optimising eDNA sample effort*

It was notable that the eDNA barcoding yielded a considerably higher number of species per site than was achieved with the gill-netting protocol, and that sampling only 9 sites (18 eDNA samples in total; total 10.8 litres of water filtered) was sufficient to reliably capture all 18 species that were recovered using the eDNA method. Collectively, this confirms the potential utility of eDNA for characterising the species community of small tropical lakes. Similar results have been achieved at the temperate Lake Windermere in the UK, where sampling eDNA from ten sites (20 litres of water filtered) was sufficient to sample the majority of the species present (Hänfling *et al.* 2016). By contrast, a study of species-rich rivers and streams in tropical French Guyana recommended that at least 68 litres of water (34 litres water from each of two replicates) would be required to inventory most of the fish species present (Cantera *et al.* 2019). The underlying causes of the differences between the volumes of water required are at present unclear. However, certainly rates of eDNA persistence differ in aquatic systems vary according to a range of environmental variables, including temperature and water chemistry (Barnes *et al.* 2014; Stickler *et al.* 2015; Seymour *et al.* 2018; Collins *et al.* 2019). Additionally, it is possible that eDNA in lotic systems does not accumulate to the same extent as in lentic systems, leading to lower detection probabilities. As studies progress, the sampling effort required to target species groups in different habitats will become clearer.

### *3.4.2 eDNA as a quantitative sampling method.*

There was a highly significant association between the number eDNA reads from a species, and the specimens captured of a species, when considering all samples. Positive associations were also seen when comparing eDNA read abundance and captures at individual sites within the lake. This is indicative of eDNA being broadly quantitative in reflecting overall abundance.

Equivalent results have been previously demonstrated in freshwater lakes (Hanfling *et al.* 2016), and marine systems (Thomsen *et al.* 2016). However, it was apparent from Lake Caobas that there were many species where reads were abundant, but captures were few or absent. For example, *Oreochromis niloticus* was extremely common in eDNA reads, yet absent from gill nets. Although it is plausible that this reflects an ability for this generalist species to avoid net-based capture methods, it may be that the species is genuinely uncommon in the lake, but exhibits a high rate of eDNA shedding. To resolve if the apparent incongruence between species abundance and eDNA read abundances can be attributed to differences in shedding rates, experiments quantifying these rates in standardised conditions reflective of the natural environment would be required (e.g. Klymus *et al.* 2015; Sassoubre *et al.* 2016).

Although the abundance of eDNA reads of a species was reflective of the relative abundance of that species in the environment, it was notable that no within-species relationships between eDNA metabarcode reads and gill net captures of that species across the sampling sites from the lake were found. This could be a consequence of eDNA of species being homogeneous across the lake, and therefore not being reflective of any fine scale habitat use of species. Alternatively, gill net captures may not be reflective of spatial abundance of species across the lake, due low catches of most species (Table 3.2). A more detailed study of abundance is required to fully evaluate fine-scale patterns of habitat use in the Lake Caobas fish community, perhaps utilising non-invasive methods such as fixed remote underwater video (Stat *et al.* 2018; Work & Jennings 2019). Equally, eDNA abundance of individual species can be evaluated using targeted species-specific probes and quantitative PCR, thereby avoiding species-level biases in PCR-based metabarcoding due to variation in amplification efficiency among templates in eDNA (Bylemans *et al.* 2018; Bylemans *et al.* 2019).

### *3.4.3 eDNA and habitat use.*

The results showed only limited evidence of community structure resolved using eDNA being associated with key habitat variables, with eDNA presence-absence of many species showing a positive association with lower pH. Spatial variation in lake pH may correlate with macrophyte presence, which was not quantified directly, and therefore variation in pH may genuinely capture habitat use of fish species. Lake Caobas has a rich macrophyte abundance in many areas, that provides shelter and are plausibly rich in prey for the benthic invertebrate and zooplankton feeders that dominate the fish assemblage (Valtierra-Vega & Schmitter-Soto 2000).

Notably fish captured using netting, were also relatively weakly associated with measured environmental variables, the exception of temperature that might reflect preferences for specific thermal regimes, or simply activity and catchability in different thermal habitats. It is worth noting that on average only approximately five species were caught in each gillnet (Figure 3.2), and the nets were dominated by the more ubiquitous zooplanktivorous species *Dorosoma petenense* and *Astyanax bacalarensis*, while most other species were rare. Thus, the absence of significant associations between the fish community structure and environmental variables may reflect a lack of power due to the relatively small catch rate in each gill net, and potential selectivity of the gillnetting approach. It is possible that longer soak times, for example over a period of 14 hours (Alexander *et al.* 2015), may have yielded a higher diversity of species that captured differential use of microhabitats by species in the lake.

### *3.4.4 Concluding remarks*

The current results illustrate the robustness of eDNA to generate an inventory of the fish species present. Our findings therefore complement those from neighbouring oligotrophic Lake Bacalar where the presence of 41 out of 57 known species in the lake and adjacent systems was resolved using eDNA (Valdez-Moreno *et al.* 2019). Importantly it is also notable that of the two compared sampling methods, only eDNA was able to reliably recover the presence the highly invasive *Oreochromis* tilapia, that potentially represents a considerable threat to the integrity of indigenous Central American fish communities (Canonico *et al.* 2005; McCrary *et al.* 2007; Deines *et al.* 2016). The results also add to the growing body of evidence that eDNA metabarcoding read counts have a weak quantitative relationship with abundance estimates (e.g. Lamb *et al.* 2019). However, at present the extent that eDNA can provide information on fine-scale habitat use in Lake Caobas is unclear, and requires further investigation using either capture-based methods, or visual censuses.

# Chapter 3

# Supplementary information

## Supplementary Information 3.1. Sample tags and primers

## Tags attached in both ends of the PCR primers during metabarcoding.

| Number | Sample | Tag | Number | Sample | Tag |
|---|---|---|---|---|---|
| 1 | A01 | TATCATT:TATCATT | 49 | E01 | CAAAGCG:CAAAGCG |
| 2 | A02 | CGGAAAC:CGGAAAC | 50 | E02 | GAGTCTA:GAGTCTA |
| 3 | A03 | TGTGGGT:TGTGGGT | 51 | E03 | CCCTGTG:CCCTGTG |
| 4 | A04 | AAACGGC:AAACGGC | 52 | E04 | AAATTCA:AAATTCA |
| 5 | A05 | GAGCTAT:GAGCTAT | 53 | E05 | GTTGAGC:GTTGAGC |
| 6 | A06 | TAGCGTG:TAGCGTG | 54 | E06 | ACCCAGC:ACCCAGC |
| 7 | A07 | GCGGGAG:GCGGGAG | 55 | E07 | CGGCTTG:CGGCTTG |
| 8 | A08 | AGCACAT:AGCACAT | 56 | E08 | AACAAAC:AACAAAC |
| 9 | A09 | TATCGCA:TATCGCA | 57 | E09 | TTAATAA:TTAATAA |
| 10 | A10 | CTCCTGA:CTCCTGA | 58 | E10 | TATTCGG:TATTCGG |
| 11 | A11 | GTTAGCA:GTTAGCA | 59 | E11 | ACCCGCA:ACCCGCA |
| 12 | A12 | AAACTTT:AAACTTT | 60 | E12 | ACAACAC:ACAACAC |
| 13 | B01 | AAAGACC:AAAGACC | 61 | F01 | CCGCTAA:CCGCTAA |
| 14 | B02 | ATCCCGG:ATCCCGG | 62 | F02 | GGTGACG:GGTGACG |
| 15 | B03 | TATCTAC:TATCTAC | 63 | F03 | AACACCA:AACACCA |
| 16 | B04 | AAAGCAT:AAAGCAT | 64 | F04 | GATAACT:GATAACT |
| 17 | B05 | GGTACCC:GGTACCC | 65 | F05 | TATTGTC:TATTGTC |
| 18 | B06 | CTGCATA:CTGCATA | 66 | F06 | CGGGCGC:CGGGCGC |
| 19 | B07 | TGTTATG:TGTTATG | 67 | F07 | AGCGGCG:AGCGGCG |
| 20 | B08 | ACATTAT:ACATTAT | 68 | F08 | GAGGAAA:GAGGAAA |
| 21 | B09 | TGTTCAC:TGTTCAC | 69 | F09 | GATACGA:GATACGA |
| 22 | B10 | GAGGCCG:GAGGCCG | 70 | F10 | AACAGGG:AACAGGG |
| 23 | B11 | CACGTAT:CACGTAT | 71 | F11 | TCCGAGG:TCCGAGG |
| 24 | B12 | CGGAGTT:CGGAGTT | 72 | F12 | TTACCTC:TTACCTC |
| 25 | C01 | GGTAGGG:GGTAGGG | 73 | G01 | GCTCAGA:GCTCAGA |
| 26 | C02 | AAAGGTA:AAAGGTA | 74 | G02 | AGCGTGC:AGCGTGC |
| 27 | C03 | TCTGTGC:TCTGTGC | 75 | G03 | TCAAATC:TCAAATC |
| 28 | C04 | CAGATCT:CAGATCT | 76 | G04 | GATAGAC:GATAGAC |
| 29 | C05 | TATGCCC:TATGCCC | 77 | G05 | AGCTAAA:AGCTAAA |
| 30 | C06 | ACCAATT:ACCAATT | 78 | G06 | ACCGCCC:ACCGCCC |
| 31 | C07 | AAAGTGG:AAAGTGG | 79 | G07 | ATGAAGA:ATGAAGA |
| 32 | C08 | CACTATA:CACTATA | 80 | G08 | CAGCAAG:CAGCAAG |
| 33 | C09 | CGGCAGT:CGGCAGT | 81 | G09 | TGACACC:TGACACC |
| 34 | C10 | TATGGAG:TATGGAG | 82 | G10 | AACCACT:AACCACT |
| 35 | C11 | AAATAGT:AAATAGT | 83 | G11 | TTACTCG:TTACTCG |

| Number | Sample | Tag | Number | Sample | Tag |
|---|---|---|---|---|---|
| 36 | C12 | CACTCCG:CACTCCG | 84 | G12 | GATATTG:GATATTG |
| 37 | D01 | AGCCCTC:AGCCCTC | 85 | H01 | TTAGAAC:TTAGAAC |
| 38 | D02 | TTAAACT:TTAAACT | 86 | H02 | GTTTGAT:GTTTGAT |
| 39 | D03 | GAGTAGC:GAGTAGC | 87 | H03 | GTCATTC:GTCATTC |
| 40 | D04 | TCTAGGA:TCTAGGA | 88 | H04 | ATCCGAC:ATCCGAC |
| 41 | D05 | AAATCTC:AAATCTC | 89 | H05 | AACCTAG:AACCTAG |
| 42 | D06 | ATCATCG:ATCATCG | 90 | H06 | ACCTACG:ACCTACG |
| 43 | D07 | AGCCGGT:AGCCGGT | 91 | H07 | CCGTATT:CCGTATT |
| 44 | D08 | ACCATAA:ACCATAA | 92 | H08 | GGTTCTT:GGTTCTT |
| 45 | D09 | AAATGAG:AAATGAG | 93 | H09 | GATCCTC:GATCCTC |
| 46 | D10 | GTCCCTA:GTCCCTA | 94 | H10 | TCACAGT:TCACAGT |
| 47 | D11 | CACTGGT:CACTGGT | 95 | H11 | AACGAGA:AACGAGA |
| 48 | D12 | TACCCAA:TACCCAA | 96 | H12 | TCTCTTG:TCTCTTG |

Forward and Reverse MiFish primers with different number of Ns and specific tags attached.

| Well Posit | Sequence Nar | Sequence Forward | Sequence Nar | Sequence Reverse |
|---|---|---|---|---|
| A01 | MiFish_F_02 | NNNTATCATTGCCGGTAAAACTCGTGCCAGC | MiFish_R_02 | NNN§CATAGTGGGGTATCTAATCCCAGTTTG |
| A02 | MiFish_F_03 | NNNNCGGAAACGCCGGTAAAACTCGTGCCAGC | MiFish_R_03 | NNCGGAAACCATAGTGGGGTATCTAATCCCAGTTTG |
| A03 | MiFish_F_04 | NNTGTGGGTGCCGGTAAAACTCGTGCCAGC | MiFish_R_04 | NNNNTGTGGGTCATAGTGGGGTATCTAATCCCAGTTTG |
| A04 | MiFish_F_05 | NNNAAACGGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_05 | NNNAAACGGCCATAGTGGGGTATCTAATCCCAGTTTG |
| A05 | MiFish_F_06 | NNNNGAGCTATGCCGGTAAAACTCGTGCCAGC | MiFish_R_06 | NNGAGCTATCATAGTGGGGTATCTAATCCCAGTTTG |
| A06 | MiFish_F_07 | NNTAGCGTGGCCGGTAAAACTCGTGCCAGC | MiFish_R_07 | NNNNTAGCGTGCATAGTGGGGTATCTAATCCCAGTTTG |
| A07 | MiFish_F_08 | NNNGCGGGAGGCCGGTAAAACTCGTGCCAGC | MiFish_R_08 | NNNGCGGGAGCATAGTGGGGTATCTAATCCCAGTTTG |
| A08 | MiFish_F_09 | NNNNAGCACATGCCGGTAAAACTCGTGCCAGC | MiFish_R_09 | NNAGCACATCATAGTGGGGTATCTAATCCCAGTTTG |
| A09 | MiFish_F_10 | NNTATCGCAGCCGGTAAAACTCGTGCCAGC | MiFish_R_10 | NNNNTATCGCACATAGTGGGGTATCTAATCCCAGTTTG |
| A10 | MiFish_F_11 | NNNCTCCTGAGCCGGTAAAACTCGTGCCAGC | MiFish_R_11 | NNNCTCCTGACATAGTGGGGTATCTAATCCCAGTTTG |
| A11 | MiFish_F_12 | NNNNGTTAGCAGCCGGTAAAACTCGTGCCAGC | MiFish_R_12 | NNGTTAGCACATAGTGGGGTATCTAATCCCAGTTTG |
| A12 | MiFish_F_13 | NNAAACTTTGCCGGTAAAACTCGTGCCAGC | MiFish_R_13 | NNNNAAACTTTCATAGTGGGGTATCTAATCCCAGTTTG |
| B01 | MiFish_F_14 | NNNAAAGACCGCCGGTAAAACTCGTGCCAGC | MiFish_R_14 | NNNAAAGACCCATAGTGGGGTATCTAATCCCAGTTTG |
| B02 | MiFish_F_15 | NNNNATCCCGGGCCGGTAAAACTCGTGCCAGC | MiFish_R_15 | NNATCCCGGCATAGTGGGGTATCTAATCCCAGTTTG |
| B03 | MiFish_F_16 | NNTATCTACGCCGGTAAAACTCGTGCCAGC | MiFish_R_16 | NNNNTATCTACCATAGTGGGGTATCTAATCCCAGTTTG |
| B04 | MiFish_F_17 | NNNAAAGCATGCCGGTAAAACTCGTGCCAGC | MiFish_R_17 | NNNAAAGCATCATAGTGGGGTATCTAATCCCAGTTTG |
| B05 | MiFish_F_18 | NNNNGGTACCCGCCGGTAAAACTCGTGCCAGC | MiFish_R_18 | NNGGTACCCCATAGTGGGGTATCTAATCCCAGTTTG |
| B06 | MiFish_F_19 | NNCTGCATAGCCGGTAAAACTCGTGCCAGC | MiFish_R_19 | NNNNCTGCATACATAGTGGGGTATCTAATCCCAGTTTG |
| B07 | MiFish_F_20 | NNNTGTTATGGCCGGTAAAACTCGTGCCAGC | MiFish_R_20 | NNNTGTTATGCATAGTGGGGTATCTAATCCCAGTTTG |
| B08 | MiFish_F_21 | NNNNACATTATGCCGGTAAAACTCGTGCCAGC | MiFish_R_21 | NNACATTATCATAGTGGGGTATCTAATCCCAGTTTG |
| B09 | MiFish_F_22 | NNTGTTCACGCCGGTAAAACTCGTGCCAGC | MiFish_R_22 | NNNNTGTTCACCATAGTGGGGTATCTAATCCCAGTTTG |
| B10 | MiFish_F_23 | NNNGAGGCCGGCCGGTAAAACTCGTGCCAGC | MiFish_R_23 | NNNGAGGCCGCATAGTGGGGTATCTAATCCCAGTTTG |
| B11 | MiFish_F_24 | NNNNCACGTATGCCGGTAAAACTCGTGCCAGC | MiFish_R_24 | NNCACGTATCATAGTGGGGTATCTAATCCCAGTTTG |
| B12 | MiFish_F_25 | NNCGGAGTTGCCGGTAAAACTCGTGCCAGC | MiFish_R_25 | NNNNCGGAGTTCATAGTGGGGTATCTAATCCCAGTTTG |
| C01 | MiFish_F_26 | NNNGGTAGGGGCCGGTAAAACTCGTGCCAGC | MiFish_R_26 | NNNGGTAGGGCATAGTGGGGTATCTAATCCCAGTTTG |
| C02 | MiFish_F_27 | NNNNAAAGGTAGCCGGTAAAACTCGTGCCAGC | MiFish_R_27 | NNAAAGGTACATAGTGGGGTATCTAATCCCAGTTTG |
| C03 | MiFish_F_28 | NNTCTGTGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_28 | NNNNTCTGTGCCATAGTGGGGTATCTAATCCCAGTTTG |
| C04 | MiFish_F_29 | NNNCAGATCTGCCGGTAAAACTCGTGCCAGC | MiFish_R_29 | NNNCAGATCTCATAGTGGGGTATCTAATCCCAGTTTG |
| C05 | MiFish_F_30 | NNNNTATGCCCGCCGGTAAAACTCGTGCCAGC | MiFish_R_30 | NNTATGCCCCATAGTGGGGTATCTAATCCCAGTTTG |
| C06 | MiFish_F_31 | NNACCAATTGCCGGTAAAACTCGTGCCAGC | MiFish_R_31 | NNNNACCAATTCATAGTGGGGTATCTAATCCCAGTTTG |
| C07 | MiFish_F_32 | NNNAAAGTGGGCCGGTAAAACTCGTGCCAGC | MiFish_R_32 | NNNAAAGTGGCATAGTGGGGTATCTAATCCCAGTTTG |
| C08 | MiFish_F_33 | NNNNCACTATAGCCGGTAAAACTCGTGCCAGC | MiFish_R_33 | NNCACTATACATAGTGGGGTATCTAATCCCAGTTTG |
| C09 | MiFish_F_34 | NNCGGCAGTGCCGGTAAAACTCGTGCCAGC | MiFish_R_34 | NNNNCGGCAGTCATAGTGGGGTATCTAATCCCAGTTTG |
| C10 | MiFish_F_35 | NNNTATGGAGGCCGGTAAAACTCGTGCCAGC | MiFish_R_35 | NNNTATGGAGCATAGTGGGGTATCTAATCCCAGTTTG |
| C11 | MiFish_F_36 | NNNNAAATAGTGCCGGTAAAACTCGTGCCAGC | MiFish_R_36 | NNAAATAGTCATAGTGGGGTATCTAATCCCAGTTTG |
| C12 | MiFish_F_37 | NNCACTCCGGCCGGTAAAACTCGTGCCAGC | MiFish_R_37 | NNNNCACTCCGCATAGTGGGGTATCTAATCCCAGTTTG |
| D01 | MiFish_F_38 | NNNAGCCCTCGCCGGTAAAACTCGTGCCAGC | MiFish_R_38 | NNNAGCCCTCCATAGTGGGGTATCTAATCCCAGTTTG |
| D02 | MiFish_F_39 | NNNNTTAAACTGCCGGTAAAACTCGTGCCAGC | MiFish_R_39 | NNTTAAACTCATAGTGGGGTATCTAATCCCAGTTTG |

| Well Posit | Sequence Nar | Sequence Forward | Sequence Nar | Sequence Reverse |
|---|---|---|---|---|
| D03 | MiFish_F_40 | NNGAGTAGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_40 | NNNNGAGTAGCCATAGTGGGGTATCTAATCCCAGTTTG |
| D04 | MiFish_F_41 | NNNTCTAGGAGCCGGTAAAACTCGTGCCAGC | MiFish_R_41 | NNNTCTAGGACATAGTGGGGTATCTAATCCCAGTTTG |
| D05 | MiFish_F_42 | NNNNAAATCTCGCCGGTAAAACTCGTGCCAGC | MiFish_R_42 | NNAAATCTCCATAGTGGGGTATCTAATCCCAGTTTG |
| D06 | MiFish_F_43 | NNATCATCGGCCGGTAAAACTCGTGCCAGC | MiFish_R_43 | NNNNATCATCGCATAGTGGGGTATCTAATCCCAGTTTG |
| D07 | MiFish_F_44 | NNNAGCCGGTGCCGGTAAAACTCGTGCCAGC | MiFish_R_44 | NNNAGCCGGTCATAGTGGGGTATCTAATCCCAGTTTG |
| D08 | MiFish_F_45 | NNNNACCATAAGCCGGTAAAACTCGTGCCAGC | MiFish_R_45 | NNACCATAACATAGTGGGGTATCTAATCCCAGTTTG |
| D09 | MiFish_F_46 | NNAAATGAGGCCGGTAAAACTCGTGCCAGC | MiFish_R_46 | NNNNAAATGAGCATAGTGGGGTATCTAATCCCAGTTTG |
| D10 | MiFish_F_47 | NNNGTCCCTAGCCGGTAAAACTCGTGCCAGC | MiFish_R_47 | NNNGTCCCTACATAGTGGGGTATCTAATCCCAGTTTG |
| D11 | MiFish_F_48 | NNNNCACTGGTGCCGGTAAAACTCGTGCCAGC | MiFish_R_48 | NNCACTGGTCATAGTGGGGTATCTAATCCCAGTTTG |
| D12 | MiFish_F_49 | NNTACCCAAGCCGGTAAAACTCGTGCCAGC | MiFish_R_49 | NNNNTACCCAACATAGTGGGGTATCTAATCCCAGTTTG |
| E01 | MiFish_F_50 | NNNCAAAGCGGCCGGTAAAACTCGTGCCAGC | MiFish_R_50 | NNNCAAAGCGCATAGTGGGGTATCTAATCCCAGTTTG |
| E02 | MiFish_F_51 | NNNNGAGTCTAGCCGGTAAAACTCGTGCCAGC | MiFish_R_51 | NNGAGTCTACATAGTGGGGTATCTAATCCCAGTTTG |
| E03 | MiFish_F_52 | NNCCCTGTGGCCGGTAAAACTCGTGCCAGC | MiFish_R_52 | NNNNCCCTGTGCATAGTGGGGTATCTAATCCCAGTTTG |
| E04 | MiFish_F_53 | NNNAAATTCAGCCGGTAAAACTCGTGCCAGC | MiFish_R_53 | NNNAAATTCACATAGTGGGGTATCTAATCCCAGTTTG |
| E05 | MiFish_F_54 | NNNNGTTGAGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_54 | NNGTTGAGCCATAGTGGGGTATCTAATCCCAGTTTG |
| E06 | MiFish_F_55 | NNACCCAGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_55 | NNNNACCCAGCCATAGTGGGGTATCTAATCCCAGTTTG |
| E07 | MiFish_F_56 | NNNCGGCTTGGCCGGTAAAACTCGTGCCAGC | MiFish_R_56 | NNNCGGCTTGCATAGTGGGGTATCTAATCCCAGTTTG |
| E08 | MiFish_F_57 | NNNNAACAAACGCCGGTAAAACTCGTGCCAGC | MiFish_R_57 | NNAACAAACCATAGTGGGGTATCTAATCCCAGTTTG |
| E09 | MiFish_F_58 | NNTTAATAAGCCGGTAAAACTCGTGCCAGC | MiFish_R_58 | NNNNTTAATAACATAGTGGGGTATCTAATCCCAGTTTG |
| E10 | MiFish_F_59 | NNNTATTCGGGCCGGTAAAACTCGTGCCAGC | MiFish_R_59 | NNNTATTCGGCATAGTGGGGTATCTAATCCCAGTTTG |
| E11 | MiFish_F_60 | NNNNACCCGCAGCCGGTAAAACTCGTGCCAGC | MiFish_R_60 | NNACCCGCACATAGTGGGGTATCTAATCCCAGTTTG |
| E12 | MiFish_F_61 | NNACAACACGCCGGTAAAACTCGTGCCAGC | MiFish_R_61 | NNNNACAACACCATAGTGGGGTATCTAATCCCAGTTTG |
| F01 | MiFish_F_62 | NNNCCGCTAAGCCGGTAAAACTCGTGCCAGC | MiFish_R_62 | NNNCCGCTAACATAGTGGGGTATCTAATCCCAGTTTG |
| F02 | MiFish_F_63 | NNNNGGTGACGGCCGGTAAAACTCGTGCCAGC | MiFish_R_63 | NNGGTGACGCATAGTGGGGTATCTAATCCCAGTTTG |
| F03 | MiFish_F_64 | NNAACACCAGCCGGTAAAACTCGTGCCAGC | MiFish_R_64 | NNNNAACACCACATAGTGGGGTATCTAATCCCAGTTTG |
| F04 | MiFish_F_65 | NNNGATAACTGCCGGTAAAACTCGTGCCAGC | MiFish_R_65 | NNNGATAACTCATAGTGGGGTATCTAATCCCAGTTTG |
| F05 | MiFish_F_66 | NNNNTATTGTCGCCGGTAAAACTCGTGCCAGC | MiFish_R_66 | NNTATTGTCCATAGTGGGGTATCTAATCCCAGTTTG |
| F06 | MiFish_F_67 | NNCGGGCGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_67 | NNNNCGGGCGCCATAGTGGGGTATCTAATCCCAGTTTG |
| F07 | MiFish_F_68 | NNNAGCGGCGGCCGGTAAAACTCGTGCCAGC | MiFish_R_68 | NNNAGCGGCGCATAGTGGGGTATCTAATCCCAGTTTG |
| F08 | MiFish_F_69 | NNNNGAGGAAAGCCGGTAAAACTCGTGCCAGC | MiFish_R_69 | NNGAGGAAACATAGTGGGGTATCTAATCCCAGTTTG |
| F09 | MiFish_F_70 | NNGATACGAGCCGGTAAAACTCGTGCCAGC | MiFish_R_70 | NNNNGATACGACATAGTGGGGTATCTAATCCCAGTTTG |
| F10 | MiFish_F_71 | NNNAACAGGGGCCGGTAAAACTCGTGCCAGC | MiFish_R_71 | NNNAACAGGGCATAGTGGGGTATCTAATCCCAGTTTG |
| F11 | MiFish_F_72 | NNNNTCCGAGGGCCGGTAAAACTCGTGCCAGC | MiFish_R_72 | NNTCCGAGGCATAGTGGGGTATCTAATCCCAGTTTG |
| F12 | MiFish_F_73 | NNTTACCTCGCCGGTAAAACTCGTGCCAGC | MiFish_R_73 | NNNNTTACCTCCATAGTGGGGTATCTAATCCCAGTTTG |
| G01 | MiFish_F_74 | NNNGCTCAGAGCCGGTAAAACTCGTGCCAGC | MiFish_R_74 | NNNGCTCAGACATAGTGGGGTATCTAATCCCAGTTTG |
| G02 | MiFish_F_75 | NNNNAGCGTGCGCCGGTAAAACTCGTGCCAGC | MiFish_R_75 | NNAGCGTGCCATAGTGGGGTATCTAATCCCAGTTTG |
| G03 | MiFish_F_76 | NNTCAAATCGCCGGTAAAACTCGTGCCAGC | MiFish_R_76 | NNNNTCAAATCCATAGTGGGGTATCTAATCCCAGTTTG |
| G04 | MiFish_F_77 | NNNGATAGACGCCGGTAAAACTCGTGCCAGC | MiFish_R_77 | NNNGATAGACCATAGTGGGGTATCTAATCCCAGTTTG |
| G05 | MiFish_F_78 | NNNNAGCTAAAGCCGGTAAAACTCGTGCCAGC | MiFish_R_78 | NNAGCTAAACATAGTGGGGTATCTAATCCCAGTTTG |
| G06 | MiFish_F_79 | NNACCGCCCGCCGGTAAAACTCGTGCCAGC | MiFish_R_79 | NNNNACCGCCCCATAGTGGGGTATCTAATCCCAGTTTG |
| G07 | MiFish_F_80 | NNNATGAAGAGCCGGTAAAACTCGTGCCAGC | MiFish_R_80 | NNNATGAAGACATAGTGGGGTATCTAATCCCAGTTTG |

| Well Posit | Sequence Nar | Sequence Forward | Sequence Nar | Sequence Reverse |
|---|---|---|---|---|
| G08 | MiFish_F_81 | NNNNCAGCAAGGCCGGTAAAACTCGTGCCAGC | MiFish_R_81 | NNCAGCAAGCATAGTGGGGTATCTAATCCCAGTTTG |
| G09 | MiFish_F_82 | NNTGACACCGCCGGTAAAACTCGTGCCAGC | MiFish_R_82 | NNNNTGACACCCATAGTGGGGTATCTAATCCCAGTTTG |
| G10 | MiFish_F_83 | NNNAACCACTGCCGGTAAAACTCGTGCCAGC | MiFish_R_83 | NNNAACCACTCATAGTGGGGTATCTAATCCCAGTTTG |
| G11 | MiFish_F_84 | NNNNTTACTCGGCCGGTAAAACTCGTGCCAGC | MiFish_R_84 | NNTTACTCGCATAGTGGGGTATCTAATCCCAGTTTG |
| G12 | MiFish_F_85 | NNGATATTGGCCGGTAAAACTCGTGCCAGC | MiFish_R_85 | NNNNGATATTGCATAGTGGGGTATCTAATCCCAGTTTG |
| H01 | MiFish_F_86 | NNNTTAGAACGCCGGTAAAACTCGTGCCAGC | MiFish_R_86 | NNNTTAGAACCATAGTGGGGTATCTAATCCCAGTTTG |
| H02 | MiFish_F_87 | NNNNGTTTGATGCCGGTAAAACTCGTGCCAGC | MiFish_R_87 | NNGTTTGATCATAGTGGGGTATCTAATCCCAGTTTG |
| H03 | MiFish_F_88 | NNGTCATTCGCCGGTAAAACTCGTGCCAGC | MiFish_R_88 | NNNNGTCATTCCATAGTGGGGTATCTAATCCCAGTTTG |
| H04 | MiFish_F_89 | NNNATCCGACGCCGGTAAAACTCGTGCCAGC | MiFish_R_89 | NNNATCCGACCATAGTGGGGTATCTAATCCCAGTTTG |
| H05 | MiFish_F_90 | NNNNAACCTAGGCCGGTAAAACTCGTGCCAGC | MiFish_R_90 | NNAACCTAGCATAGTGGGGTATCTAATCCCAGTTTG |
| H06 | MiFish_F_91 | NNACCTACGGCCGGTAAAACTCGTGCCAGC | MiFish_R_91 | NNNNACCTACGCATAGTGGGGTATCTAATCCCAGTTTG |
| H07 | MiFish_F_92 | NNNCCGTATTGCCGGTAAAACTCGTGCCAGC | MiFish_R_92 | NNNCCGTATTCATAGTGGGGTATCTAATCCCAGTTTG |
| H08 | MiFish_F_93 | NNNNGGTTCTTGCCGGTAAAACTCGTGCCAGC | MiFish_R_93 | NNGGTTCTTCATAGTGGGGTATCTAATCCCAGTTTG |
| H09 | MiFish_F_94 | NNGATCCTCGCCGGTAAAACTCGTGCCAGC | MiFish_R_94 | NNNNGATCCTCCATAGTGGGGTATCTAATCCCAGTTTG |
| H10 | MiFish_F_95 | NNNTCACAGTGCCGGTAAAACTCGTGCCAGC | MiFish_R_95 | NNNTCACAGTCATAGTGGGGTATCTAATCCCAGTTTG |
| H11 | MiFish_F_96 | NNNNAACGAGAGCCGGTAAAACTCGTGCCAGC | MiFish_R_96 | NNAACGAGACATAGTGGGGTATCTAATCCCAGTTTG |
| H12 | MiFish_F_01 | NNTCTCTTGGCCGGTAAAACTCGTGCCAGC | MiFish_R_01 | NNNNTCTCTTGCATAGTGGGGTATCTAATCCCAGTTTG |

**Supporting Information 3.2: eDNA extraction protocol**

For DNA extractions the DNeasy Blood & Tissue Kit (Qiagen) was used. The filter paper from the Sterivex filter unit was placed into a 1.5ml tube. Then 180 µl of ATL buffer and 20 µl proteinase K were added to the sample, before mixing by vortexing, and incubation at 56°C. Occasional vortexing during incubation helped to ensure complete lysis of material on filters. When lysis was complete, 200 µl of AL buffer was added, and the sample thoroughly mixed by vortexing, before samples were again incubated at 56°C for 10 minutes. Next, 200 µl of laboratory grade ethanol was added to the sample, which was then mixed thoroughly by vortexing. This mixture was then pipetted into a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute, after which the flow-through and collection tube were discarded. The spin column was then placed in a new 2 ml collection tube and added 500 µl of AW1 buffer, and again centrifuged again for 1 minute at 8000 rpm, before discarding the flow-through and collection tube. Then, the spin column was placed in a new 2 ml collection tube, 500 µl AW2 buffer added, and then centrifuged for 3 minutes at 14,000 rpm. Again, the flow-through and collection tube were then discarded. The spin column was then placed into a 2 ml microcentrifuge tube, and the DNA eluted by adding 200 µl of AE buffer to the centre of the spin column membrane. The sample was incubated for 1 minute at room temperature (15 – 25°C), and then centrifuged for 1 minute at 8000 rpm. To increased DNA yield, the eluted material was then placed back into the spin column, again incubated for 1 minute and centrifuged for 1 minute at 8000 rpm. Samples were then stored in a dedicated eDNA freezer at -20°C.

**Supporting Information 3.3: Tissue DNA extraction protocol**

DNA samples (fin clip) were incubated with nuclei lysis solution (200ml) and proteinase K (5 ml) at 56°C, for at least 30 minutes or until the tissue was lysed entirely, vortexing the samples periodically. The samples were then centrifuged for 2 minutes at 14000 rpm. The supernatant was then transferred to a new 1.5 ml tube, 65 ml of protein precipitation solution added, and the samples vortexed before being centrifuged at 14000 rpm for 2 minutes. The supernatant was then transferred to a new 1.5 ml tube and DNA precipitated by adding 600 ml of 100% ethanol. After approximately 20 inversions, the samples were centrifuged at 14000 rpm for 2 minutes; the ethanol was then removed. Finally, the samples were washed, adding 600 ml of 70% cold ethanol; then, the samples were centrifuged again at 14000 rpm for 2 minutes. The samples were then left to dry at room temperature for 8 hours, before being resuspended in 50 ml of elution buffer (Qiagen AE buffer, part no. 19077), and finally, store the DNA samples at -20°C.

**Supporting Information 3.4: Bioinformatic script**

Core script can be found using the following link:

https://github.com/boopsboops/crack-pipe

The script was adapted to work with the files obtained from the Illumina sequencer for the present work.

After preparing the machine and the working area to perform the bioinformatic work the following step was merging the sequences giving the following commands:

```
#!/usr/bin/env sh

while getopts t:f:r: option
do
case "${option}"
in
f) R1=${OPTARG};;
r) R2=${OPTARG};;
t) THREADS=${OPTARG};;
esac
done

# run vsearch
vsearch --threads "$THREADS" --fastq_mergepairs "$R1" --reverse "$R2" --
fastqout temp/merged/merged.fastq
printf "...\n...\n...\nJust zipping up ...\n"
gzip -f temp/merged/merged.fastq

# run pear and gzip results
#pear -j "$THREADS" -f "$R1" -r "$R2" -o temp/merged/reads
#gzip -f temp/merged/reads.assembled.fastq
#printf "...\n...\n...\nFinished!\n"
# ./merge-reads.sh -f temp/fastq/12S-mifishu-R1.fastq.gz -r temp/fastq/12S-
mifishu-R2.fastq.gz
The following step was to reorientate the reads through the following
commands:
#!/usr/bin/env sh
# set params #
while getopts f:r:m:n: option
do
case "${option}"
in
f) FWD=${OPTARG};;
r) REV=${OPTARG};;
m) MINLENF=${OPTARG};;
n) MINLENR=${OPTARG};;
esac
done
# extract fwd and rev
cutadapt --error-rate 0.15 --overlap "$MINLENF" --action=none -g fwd="$FWD"
--untrimmed-output temp/trash/nofwd.fastq.gz -o temp/trash/fwd.fastq.gz
temp/merged/merged.fastq.gz
cutadapt --error-rate 0.15 --overlap "$MINLENR" --action=none -g rev="$REV"
--untrimmed-output temp/trash/norev.fastq.gz -o temp/trash/rev.fastq.gz
temp/trash/nofwd.fastq.gz

# revcomp the rev
```

```
vsearch --fastx_revcomp temp/trash/rev.fastq.gz --fastqout
temp/trash/rev.revcomp.fastq
gzip -f temp/trash/rev.revcomp.fastq
# join
cat temp/trash/fwd.fastq.gz temp/trash/rev.revcomp.fastq.gz >
temp/reorientated/reorientated.fastq.gz
printf "...\n...\n...\nFinished!\n"
# ./reorientate.sh -f GTCGGTAAAACTCGTGCCAGC -r CATAGTGGGGTATCTAATCCCAGTTTG
-m 21 -n 27
```

Then the workflow continued with the demultiplexing through the following commands:

```
#!/usr/bin/env sh
# set params #
while getopts t:f:r: option
do
case "${option}"
in
f) FWD=${OPTARG};;
r) REVCOMP=${OPTARG};;
t) THREADS=${OPTARG};;
esac
done
# split the FASTQ
# create tmp folders
#THREADS=8
for i in `seq 1 "$THREADS"`; do
mkdir temp/demultiplexed/reorientated.part_00"$i"
done
# split the files
# https://bioinf.shenwei.me/seqkit/
seqkit split2 -f temp/reorientated/reorientated.fastq.gz -p "$THREADS"
# get file names - also see `basename`
FILES="$(ls temp/reorientated/reorientated.fastq.gz.split/*.fastq.gz | sed
-e 's/\.fastq\.gz//g' | sed -e
's/temp\/reorientated\/reorientated.split\///g')"
# run demultiplex loop
for i in $FILES; do
cutadapt --no-indels --error-rate 0.1 --overlap 10 --action=none -g
file:temp/fastq/barcodes.fas -o temp/demultiplexed/"$i"/{name}.fastq.gz --
discard-untrimmed
temp/reorientated/reorientated.fastq.gz.split/"$i".fastq.gz &
done; wait
# now cat all of the files back into the same files -
find temp/demultiplexed -type f -name "*.fastq.gz" | while read F; do
basename ${F%.fastq.gz}; done | sort | uniq | while read P; do find
temp/demultiplexed -type f -name "${P}*.fastq.gz" -exec cat '{}' ';' >
temp/demultiplexed/${P}.merged.fastq.gz; done
# trim with cutadapt
FILES="$(ls temp/demultiplexed/*.fastq.gz | sed -e 's/\.fastq\.gz//g' | sed
-e 's/temp\/demultiplexed\///g')"
# check
#for j in $FILES; do echo "$j"; done
# now run
for i in $FILES; do
cutadapt -n 5 --error-rate 0.15 -g "$FWD" --discard-untrimmed
temp/demultiplexed/"$i".fastq.gz | cutadapt -n 5 --error-rate 0.15 -a
"$REVCOMP" -o temp/trimmed/"$i".fastq.gz --discard-untrimmed - &
done; wait
printf "...\n...\n...\nFinished!\n"
# ./demultiplex.sh -t 8 -f GTCGGTAAAACTCGTGCCAGC -r
CAAACTGGGATTAGATACCCCACTATG
```

Afterwards, the workflow continued with the dereplication step:

```
#!/usr/bin/env sh
# set params #
while getopts e:a:p: option
do
case "${option}"
in
a) AVG=${OPTARG};;
p) PROP=${OPTARG};;
e) MEE=${OPTARG};;
esac
done
MINLEN=$(awk "function ceil(x, y){y=int(x); return(x>y?y+1:y)} BEGIN {
pc=${AVG}-${AVG}*${PROP}; i=ceil(pc); print i }")
MAXLEN=$(awk "function ceil(x, y){y=int(x); return(x>y?y+1:y)} BEGIN {
pc=${AVG}+${AVG}*${PROP}; i=ceil(pc); print i }")
# qc filter
FILES="$(ls temp/trimmed/*.fastq.gz | sed --
expression='s/\.merged\.fastq\.gz//g' | sed --
expression='s/temp\/trimmed\///g')"
# check
#for f in $FILES; do echo "$f"; done
# now run
for i in $FILES; do
    vsearch --fastq_filter temp/trimmed/"$i".merged.fastq.gz --fastq_maxee
"$MEE" --fastq_minlen "$MINLEN" --fastq_maxlen "$MAXLEN" --fastq_maxns 0 --
fastaout temp/filtered/"$i".fasta --fasta_width 0 &
done; wait
# derep
FILES="$(ls temp/filtered/*.fasta | sed --expression='s/\.fasta//g' | sed -
-expression='s/temp\/filtered\///g')"
# check
#for f in $FILES; do echo "$f"; done
# now run
for i in $FILES; do
    vsearch --derep_fulllength temp/filtered/"$i".fasta --minuniquesize 1 -
-output temp/dereplicated/"$i".fasta --relabel_md5 --sizeout --fasta_width
0 &
done; wait
#printf "...\n...\n...\nFinished!\n"
printf "...\nMinimum length of fragment is: $MINLEN bp\n"
printf "...\nMaximum length of fragment is: $MAXLEN bp\n"
# ./dereplicate.sh -a 170 -p 0.15
```

Then the cluster of the reads continued:

```
#!/usr/bin/env sh
# set params #
while getopts t:u: option
do
case "${option}"
in
u) UNIQPROP=${OPTARG};;
t) THREADS=${OPTARG};;
esac
done
# cat all fasta
cat temp/dereplicated/*.fasta > temp/clustered/combined.derep.fasta
# gloabl derep
vsearch --derep_fulllength temp/clustered/combined.derep.fasta --sizein --
sizeout --fasta_width 0 --output temp/clustered/combined.glob.derep.fasta
# swarm
swarm -t "$THREADS" -d 1 -z -f -o temp/clustered/swarm.clusters.out -w
temp/clustered/swarm.clusters.fasta
temp/clustered/combined.glob.derep.fasta
```

```
# Sort representatives
vsearch --fasta_width 0 --sortbysize temp/clustered/swarm.clusters.fasta --
output temp/clustered/swarm.clusters.sorted.fasta
# chimaera search
vsearch --fasta_width 0 --uchime_denovo
temp/clustered/swarm.clusters.sorted.fasta --uchimeout
temp/clustered/swarm.cleaned.uchime --nonchimeras
temp/clustered/swarm.cleaned.fasta
# get nreads
NREADS=$(grep ";size=" temp/clustered/swarm.cleaned.fasta | sed -e
's/.*;size=//g' | awk '{ SUM += $1} END { print SUM }')
DISCARD=$(awk "function ceil(x, y){y=int(x); return(x>y?y+1:y)} BEGIN {
pc=${NREADS}*${UNIQPROP}; i=ceil(pc); print i }")
# remove ntons
vsearch --derep_fulllength temp/clustered/swarm.cleaned.fasta --sizein --
sizeout --fasta_width 0 --minuniquesize "$DISCARD" --output
results/cleaned-reads.fasta
# homology search
#hmmsearch -E 0.01 --incE 0.01 hmms/12s.miya.primers.hmm
temp/clustered/swarm.cleaned.rmsingletons.fasta | grep ">> " | sed -e 's/>>
//g' -e 's/[[:space:]]//g' | sort | uniq > temp/clustered/hmm-out.txt
#sed -e 's/;size=[0-9]*//g' temp/clustered/swarm.clusters.out | nl -w 1 |
sed -e 's/^/swarm/g' -e 's/ /\t/g' > temp/clustered/swarm.clusters.tsv
sed -e 's/;size=[0-9]*//g' temp/clustered/swarm.clusters.out >
temp/clustered/swarm.clusters.tsv
# report
printf "...\n...\n...\nSequences with fewer than $DISCARD reads have been
discarded\n"
#./cluster.sh -t 8 -u 5
```

Then, the workflow opens the R application to assemble the reference library to the sequences:

```
#!/usr/bin/env Rscript
suppressMessages(library("rfishbase"))
suppressMessages(library("ape"))
suppressMessages(library("tidyverse"))
suppressMessages(library("parallel"))
suppressMessages(library("magrittr"))
data(fishbase)

# read in refs
custom.refs <- read.FASTA(file="temp/reference-library/custom-
references.fasta")

# get unique names
custom.df <-
tibble(code=str_split_fixed(names(custom.refs),"\\|",2)[,1],sciName=str_spl
it_fixed(names(custom.refs),"\\|",2)[,2]) %>%
    mutate(Genus=str_split_fixed(sciName," ",2)[,1])

# add full sci name to fishbase
#fishbase %<>% mutate(sciName=paste(Genus,Species))

# check names validity
print("Following genera are not in FishBase db. Spelling error, or maybe a
try a synonym? 'character(0)' means all genera were found in FishBase.")
setdiff(unique(pull(custom.df,Genus)),fishbase$Genus)
fishbase %<>% filter(Genus %in% unique(pull(custom.df,Genus)))
#rfishbase::synonyms(setdiff(names.unique,fishbase$sciName))

# make taxonomy
fishbase %<>% mutate(kingdom="Animalia",phylum="Chordata") %>%
    select(kingdom,phylum,Class,Order,Family,Genus) %>%
```

```
    distinct()

# combine
custom.df <- suppressMessages(left_join(custom.df, fishbase)) %>%
    filter(!is.na(kingdom)) %>%
    mutate(label=paste0(code,";tax=k:",kingdom,",p:",phylum,",c:",Class,",o
:",Order,",f:",Family,",g:",Genus,",s:",sciName)) %>%
    mutate(label=str_replace_all(label," ","_"))

# trim names and remove non-matched
names(custom.refs) <- str_split_fixed(names(custom.refs),"\\|",2)[,1]
custom.refs.sub <- custom.refs[which(names(custom.refs) %in%
pull(custom.df,code))]

# add new names
names(custom.refs.sub) <-
pull(custom.df,label)[match(names(custom.refs.sub), pull(custom.df,code))]

# write out
write.FASTA(custom.refs.sub,file="temp/reference-library/custom-references-
annotated.fasta")
Then, the assignation of the taxonomy was the following step:
 #!/usr/bin/env sh
# set params #
while getopts a:p:t:c: option
do
case "${option}"
in
c) CUTOFF=${OPTARG};;
t) THREADS=${OPTARG};;
a) AVG=${OPTARG};;
p) PROP=${OPTARG};;
esac
done
# calculate minlen
MINLEN=$(awk "function ceil(x, y){y=int(x); return(x>y?y+1:y)} BEGIN {
pc=${AVG}*${PROP}; i=ceil(pc); print i }")
# trim primers from the custom reference library
cutadapt -n 1 -e 0.3 -O 10 -g GTCGGTAAAACTCGTGCCAGC temp/reference-
library/custom-references-annotated.fasta | cutadapt --minimum-length
"$MINLEN" -n 1 -e 0.3 -O 10 -a CAAACTGGGATTAGATACCCCACTATG -o
temp/reference-library/custom-references-annotated.trimmed.fasta -
# merge with refseq
cat temp/reference-library/custom-references-annotated.trimmed.fasta
assets/refseq-mtdna-with-taxonomy.fasta > results/reference-library.fasta
# run sintax
vsearch --threads "$THREADS" --sintax results/cleaned-reads.fasta --db
results/reference-library.fasta --sintax_cutoff "$CUTOFF" --tabbedout
results/taxonomy-assignments.tsv
# report
printf "...\n...\n...\nMinimum length of fragment is: $MINLEN bp\n"
#./assign-taxonomy.sh -t 8 -a 170 -p 0.7 -c 0.7
Afterwards, making the OTU tables was the following step:
#!/usr/bin/env Rscript
suppressMessages(library("ape"))
suppressMessages(library("tidyverse"))
suppressMessages(library("parallel"))
suppressMessages(library("magrittr"))
# ./make-otu-table.R
# load drep fasta and clean
fas.list <- list.files(path="temp/dereplicated",pattern=".fasta")
```

```
fas.all <- mcmapply(function(x) read.FASTA(file=x),
paste0("temp/dereplicated/",fas.list), mc.cores=4)
names(fas.all) <- str_replace_all(fas.list,".fasta","")
# extract names and adundances per sample
samples.tabulated <- mcmapply(function(x,y)
tibble(md5=str_split_fixed(names(x),";size=",2)[,1],
size=str_split_fixed(names(x),";size=",2)[,2], sample=y), fas.all,
names(fas.all), mc.cores=4, SIMPLIFY=FALSE)
samples.tabulated.joined <- bind_rows(samples.tabulated)
# load up swarm results
swarms <- read_lines(file="temp/clustered/swarm.clusters.tsv",)
mothers <- mcmapply(function(x) x[1], str_split(swarms," "), mc.cores=4)
daughters <- mcmapply(function(x) paste(x[-1],collapse=" "),
str_split(swarms," "), mc.cores=4)
# make a nested table of daughters and then flatten
daughters.unlist <- mcmapply(function(x) unlist(str_split(x,"
",simplify=FALSE)), daughters, mc.cores=4, USE.NAMES=FALSE)
all.swarms <- tibble(mother=mothers, daughter=daughters.unlist) %>%
unnest()
# collapse and join by sample
samples.merged <- samples.tabulated.joined %>%
    mutate(mother=all.swarms$mother[match(md5,all.swarms$daughter)]) %>%
    mutate(mother=if_else(is.na(mother),md5,mother)) %>%
    group_by(sample,mother) %>%
    summarise(sum=sum(as.numeric(size))) %>%
    ungroup()
# load up the final cleaned reads
keeps <- read.FASTA(file="results/cleaned-reads.fasta")
keeps.names <- str_replace_all(names(keeps),";size=[0-9]*","")
# filter just good seqs
samples.kept <- samples.merged %>% filter(mother %in% keeps.names)
# check numbers are the same
print("read numbers are the same?")
samples.kept %>% pull(sum) %>% sum ==
sum(as.numeric(str_replace_all(names(keeps),".*;size=","")))
# create an otu table and write out
samples.kept %>% spread(key=sample,value=sum,fill=0) %>%
    write_csv(path="results/otu-table-raw.csv")
# read in taxonomy assignment
tax.ass.df <-
suppressMessages(suppressWarnings(read_tsv(file="results/taxonomy-
assignments.tsv",col_names=c("md5","idsProbs","strand","ids"))))
# clean md5 and extract best IDs
tax.ass.df %<>% mutate(md5=str_replace_all(md5,";size=[0-9]*",""),
bestId=str_replace_all(map(str_split(tax.ass.df$ids,":"), last),"_"," "))
%>%
    mutate(isFish=if_else(grepl("Cephalaspidomorphi",idsProbs) |
grepl("Elasmobranchii",idsProbs) | grepl("Actinopterygii",idsProbs), TRUE,
FALSE))
# match to OTU table
assigned.all <- samples.kept %>%
mutate(assignment=pull(tax.ass.df,bestId)[match(mother,pull(tax.ass.df,md5)
)]) %>%
    mutate(isFish=pull(tax.ass.df,isFish)[match(mother,pull(tax.ass.df,md5)
)]) %>%
    mutate(assignment=if_else(is.na(assignment),"unassigned",assignment))
# collapse by ID
assigned.all %>%
    group_by(sample,assignment) %>%
    summarise(sum=sum(sum)) %>%
    ungroup() %>%
    spread(key=sample,value=sum,fill=0) %>%
```

```
    write_csv(path="results/otu-table-all.csv")
# fish only, and collapse by all
assigned.all %>%
    filter(isFish==TRUE) %>%
    group_by(sample,assignment) %>%
    summarise(sum=sum(sum)) %>%
    ungroup() %>%
    spread(key=sample,value=sum,fill=0) %>%
    write_csv(path="results/otu-table-fish.csv")
# get total number fish reads
assigned.all %>%
    filter(isFish==TRUE) %>%
    pull(sum) %>%
    sum() %>%
    write("temp/reference-library/nfishreads.txt")
```

The following step was to generate the statistics to know the how the number of reads were changing through the pipeline:

```
#!/usr/bin/env sh
while getopts f: option
do
case "${option}"
in
f) R1=${OPTARG};;
esac
done
printf "Total number raw reads:\n"
seqkit stats -b "$R1"
printf "\n\n"
printf "Total number merged reads:\n"
seqkit stats -b temp/merged/merged.fastq.gz
printf "\n\n"
printf "Total number reorientated reads:\n"
seqkit stats -b temp/reorientated/reorientated.fastq.gz
printf "\n\n"
printf "Total number demultiplexed reads:\n"
cat temp/demultiplexed/*.fastq.gz | seqkit stats
printf "\n\n"
printf "Total number trimmed reads:\n"
cat temp/trimmed/*.fastq.gz | seqkit stats
printf "\n\n"
printf "Total number filtered reads:\n"
cat temp/filtered/*.fasta | seqkit stats
printf "\n\n"
printf "Total number cleaned reads:\n"
grep ";size=" results/cleaned-reads.fasta | sed -e 's/.*;size=//g' | awk '{
SUM += $1} END { print SUM }'
printf "\n\n"
printf "Total number fish reads:\n"
cat temp/reference-library/nfishreads.txt
printf "\n\n"
# ./generate-stats.sh -f temp/fastq/12S-mifishu-R1.fastq.gz
```

If it is important to run any parts of the pipeline again, it is possible to do it from any point, but it is safest to first delete and recreate any previously populated directories from that step. Then the OTU table is ready to be analysed according to the aims of the research project.

# Chapter 4

# Indigenous fish community structure and Nile tilapia abundance in freshwater lakes of southern Mexico

An adapted version of this chapter is in preparation to be submitted to a peer-reviewed journal:

Gracida Juarez C.A. and Genner, M.J. Indigenous fish community structure and Nile tilapia abundance in freshwater lakes of southern Mexico

Author contributions: CAGJ and MJG designed the study. CAGJ conducted the field survey. CAGJ and MJG conducted the statistical analyses. CAGJ led the writing of the manuscript. MJG contributed critically to drafts.

**Abstract**

Increased aquaculture production has resulted in the widespread establishment of African tilapia of the genus *Oreochromis* in Mexican freshwaters, and there are concerns that this invasive species may negatively affect native fish biodiversity. However, there is still limited quantitative evidence of the abundance of *Oreochromis* relative to native species in the region, or the factors that influence fish diversity. In this study the ichthyofauna of six lakes in the state of Quintana Roo on the Yucatan Peninsula were quantified. *Oreochromis* was sampled from only three of the six lakes studied, and in each of those lakes tilapia represented less than 3% of the total fish captured. Moreover, no evidence was found that the presence of tilapia in a lake was a major influence on native fish biodiversity. Instead, total fish biodiversity was most strongly predicted by oxygen availability, with more oxygenated conditions associated with the highest biodiversity both within and across lakes. Spatial comparisons also highlighted the importance of other water chemistry variables, alongside substrate heterogeneity and lake area, as influential in determining native fish community structure. On the basis of these results, the tilapia populations of these lakes may have strong limitations on their size, either due to the presence of predatory native fish species, or limited suitable ecological resources (food, breeding habitat). Hence, conservation-orientated lake-wide management of native species and their core habitats may be key to avoiding negative effects of tilapia expansions in the region.

## 4.1 Introduction

Biological invasions are problematic due to their often-substantive harmful impact on national economies, and the substantive ecological change that can be caused by negative interactions with native species (Crowl *et al.* 2008). Such interactions include predation on native species, competition with native species for limited resources, and the ability of invasive species to act as vectors of disease that infect native species (Marsh & Douglas 1997; Hobbs 2000; Bergstrom & Mensinger 2009; Harrison *et al.* 2013). In freshwaters, invasive species are considered among the major main drivers of species loss, alongside habitat loss, pollution and overexploitation (Vörösmarty *et al.* 2010). However, there is still a relatively poor understanding of the biology of many of these invasive species, and specifically why the success of invasive species varies spatially and temporally within their invaded ranges. Most commonly this is viewed from the perspective of physiologically-determined thermal limitations (Peterson 2003), however a greater understanding of other ecological factors that determine the success of invasive species may be beneficial for understanding their ultimate impacts on the natural environment and human economies that depend upon them.

Tilapia are a non-monophyletic group of African cichlid fishes (Chapter 1) that are now utilised in aquaculture in more than 100 countries across tropical and subtropical regions of the world (Deines *et al.* 2016). The most commonly used species are those of the mouthbrooding genus *Oreochromis,* which are characterised by fast growth rates, a high food conversion ratio, tolerance to high densities, disease resistance, and ability to thrive in relatively low oxygen concentration (Vicente & Fonseca-Alves, 2013). However, those traits also favour the invasive success of the *Oreochromis* in natural water bodies, and they have been implicated in leading to declines in native species, primarily through competition for limited resources (Canonico

*et al.* 2005). For example, in Lake Nicaragua the Nile tilapia (*Oreochromis niloticus*) has driven a 54% decline in catches of native cichlids that are important for local fisheries (McKaye *et al.* 1995). In Mexico, several species of tilapia have been introduced into natural water bodies, through a combination of deliberate introductions in attempts to enhance capture fisheries, or accidental introductions following escapes from aquaculture facilities (Schmitter-Soto & Caro 1997). Notably, transfers appear to take place through superficial streams which are common during heavy rainy seasons and are used as mechanism of dispersion (Esselman *et al.* 2013).

In the state of Quintana Roo, on the Yucatan Peninsula of Mexico, tilapia-based aquaculture has been growing steadily over recent decades. The most recent surveys of natural freshwaters in the region conducted in the 1990s demonstrated a broad distribution of tilapia (Schmitter-Soto & Caro 1997) . The present survey aimed to update the evidence regarding the current abundance of invasive tilapia in a set of six lakes. It also aimed to gain a better understanding of how tilapia and environmental variables may together, or independently, affect native fish assemblages. The results demonstrate an overall low abundance of tilapia relative to native species and indicate that invasive species are not currently key drivers of the community structure of indigenous fishes in the sampled lakes. Instead, there is evidence to suggest that physiochemical environmental variables are drivers of community structure. The results are discussed from the perspective of the importance factors that may limit the success of tilapia in the surveyed lakes.

## *4.2 Methods*

### *4.2.1 Study sites.*

Six lakes were sampled in the state of Quintana Roo, on the Yucatan Peninsula, Mexico. The lakes were selected based on the reported presence of *Oreochromis* in three of them and its absence in the other three (Figure 4.1, Table 4.1). The water in these lentic systems is rich in calcium due to the calcareous nature of the local terrain. These six permanent lakes can increase in volume up to 150% during the rainy season which starts in May and ends in October, with the highest precipitation (average: 202 mm) occurring between June and October. The highest air temperatures are in April and May, typically between 22.6°C and 36.0°C (average: 28.5°C), while the lowest temperature are between December and February, typically between 15.5°C and 24.5°C (average: 19.5°C). During July, which is the month when the present survey took place, temperatures typically range between 21.3°C and 31.2°C (average: 26°C) (Data from: WorldWeatherOnline.com, consulted 19/02/2020).

The aquatic vegetation within the lakes is dominated by the genera *Nymphoides*, *Potamogeton* and *Typha* (Trejo-Torres *et al.* 1993). The lake substrate varies among and within lakes, comprising mud, sand, gravel or calcareous rocks. Typically, the water in the lakes is transparent near the shore, but becomes increasingly turbid towards the centre.

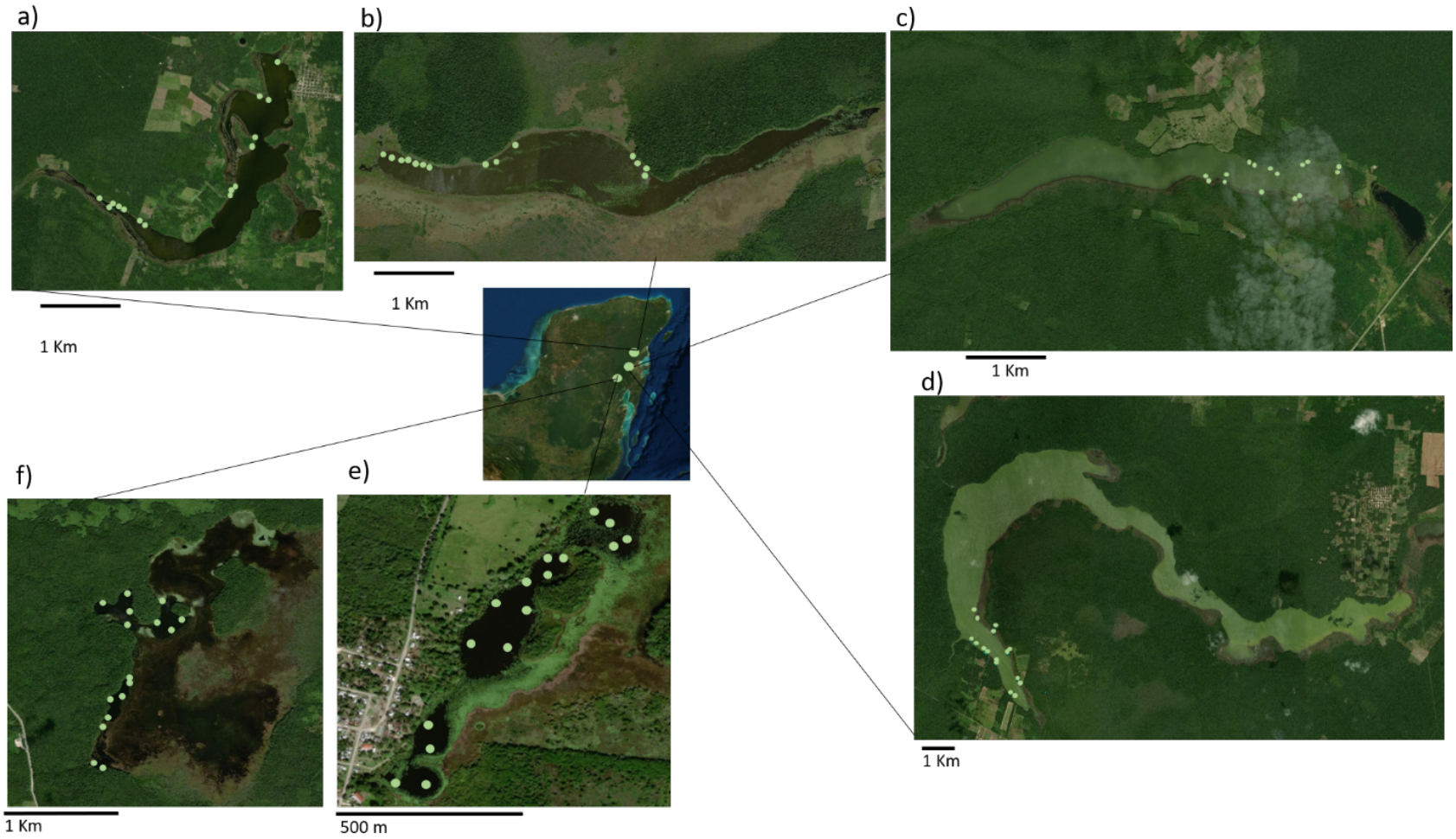


**Figure 4.1.** Lakes sampled. a) Nohbec, b) Petcacab, c) Sta Teresita, d) San Felipe, e) Caobas, f) Chacanbacan. Filled circles indicate sampling locations in each lake.

**Table 4.1.** Details of the six surveyed freshwater lakes. Pair 1: Nonbec-Petcacab, Pair 2: Sta Teresita-San Felipe, Pair 3: Caobas-Chacambacan.

| Variable [Code in analyses] | Nohbec | Petcacab | Sta Teresita | San Felipe | Caobas | Chacambacan |
|---|---|---|---|---|---|---|
| Latitude | 19° 08' 00.62'' | 19° 11' 54.58'' | 18° 54' 46.55'' | 18° 46' 19.67'' | 18° 26' 59.56'' | 18° 29' 29.21'' |
| Longitude | 88° 10' 53.88'' | 88° 27' 34.82'' | 88° 14' 52.33'' | 88° 28' 13.56'' | 89° 05' 53.71'' | 89° 05' 01.24'' |
| Survey date | 11-12 July 2018 | 13-14 July 2018 | 17-18 July 2018 | 19-20 July 2018 | 23-24 July 2018 | 25-26 July 2018 |
| Depth (mean, m)* | 151.8 | 160.6 | 195.6 | 168.8 | 205.2 | 311.25 |
| Transparency (mean, cm) * | 118.75 | 93.75 | 99.37 | 82.50 | 98.75 | 133.13 |
| Distance to shore (mean, m)* | 30.62 | 37.00 | 33.31 | 29.50 | 18.75 | 26.06 |
| pH (mean, pH units)* | 7.83 | 7.51 | 7.51 | 7.82 | 8.14 | 8.04 |
| Dissolved oxygen (mean, mg/l)* | 4.18 | 4.63 | 4.09 | 5.12 | 5.89 | 5.51 |
| Temperature (°C)* | 31.03 | 31.08 | 31.78 | 32.05 | 31.61 | 30.96 |
| Area ($m^2$) [Area] | 9,722,476 | 3,192,165 | 1,699,604 | 17,250,353 | 129,340 | 353,105 |
| Substrate complexity** | 2 | 1 | 2 | 1 | 3 | 3 |

*Average data obtained in the field for the 16 sampling locations; **ordinal scale of increasing complexity, see methods for details, ***in this survey. Pairs of geographically-proximate freshwater lakes: Pair 1) Nonbec-Petcacab, Pair 2) Sta Teresita-San Felipe, Pair 3) Caobas-Chacambacan.

### *4.2.2 Data collection.*

Sampling took place between the 11th and 26th of July of 2018, between the 7 and 18 hours. Sixteen sampling points were surveyed per lake, eight points per day. Sampling was conducted using survey gill nets (CEN Standard Multi Mesh 30 m long x with a stretched depth of 1.5 m with twelve panels of 2.5 m long each, in the following order: 43 mm, 19.5 mm, 6.25 mm, 10 mm, 55 mm, 8 mm, 12.5 mm, 24 mm, 15.5 mm, 5 mm, 35 mm, and 29 mm monofilament). Each gear set was for a duration of one hour, and specimens removed from the net. To enable identifications, each specimen was assigned a code, photographed and preserved in 100% ethanol. Scientific names were corroborated following Frickle *et al.* (2019).

At each sampling point water temperature and pH were measured using a pH-meter (ExStik™, China); dissolved oxygen was measured using a portable DO-meter (HI-9146, Hanna Instruments, Romania), and depth and water transparency were measured using a secchi disc (310 mm in diameter). Coordinates of each sampling site were taken using a handheld geographic positioning system (GPS) (Garmin Corp, Lenexa, Kansas, USA). The distance from sampling point to the shore and area of each aquatic system were calculated in using Google Earth. A value from 1 to 3 was assigned to the substrate of each lake according to the Unified Soil Classification System (ASTM, 2011) and following Neves *et al.* (2013).

### *4.2.3 Data analysis - Within lake diversity*

For each lake, from all 16 sampling events, fish species diversity and abundance metrics were calculated, including the total species richness, frequency of sampling events in which a species was represented (%F), and the mean abundance within each sampling event (A). An

individual-based rarefaction method was used to estimate how the number of species encountered was dependent on sampling effort, which was calculated using the specaccum function from package Vegan in R (Oksanen *et al.,* 2017).

### *4.2.4 Data analysis – comparisons of community structure across lakes*

To illustrate the differences in fish communities among lakes, non-metric multidimensional scaling (nMDS) was used on $\log_{10}(x+1)$ transformed species abundance data using the Bray-Curtis similarity index and the metaMDS function in the R package "Vegan" (Oksanen *et al.,* 2017). To test for differences in the fish communities among lakes, Permanova was used with on $\log_{10}(x+1)$ transformed abundance data and the Bray-Curtis similarity index, employing 10,000 permutations, using the adonis function in R package "Vegan". To identify the species that contribute the most to dissimilarity of the lake fish communities, similarity percentage analysis (SIMPER; Clarke, 1993) was used with $\log_{10}(x+1)$ transformed abundance data and the Bray-Curtis similarity index, using the "pool all groups" option in PAST4 (Hammer *et al.* 2001).

To quantify how environmental variables associate with fish diversity across all lakes, a Canonical Correspondence Analysis (RDA) was used, with $\log_{10}(x+1)$ transformed abundance data with the capscale function in the R package "Vegan". The variance explained by each variable was then quantified using the Anova-like permutation test for RDA in the R package "Vegan", using the by "margin" option and 10,000 permutations. Then, the varpart variance-partitioning approach in the "Vegan" package was used to determine the combined relative contribution of each of the following three sets of environmental variables to explain differences in the fish communities: a) local *in-situ* environmental variables (i.e. water chemistry, depth), b) lake specific variables (lake area, dominant substrate), and c) presence or absence of *Oreochromis* tilapia in the lake.

## 4.3 Results

### 4.3.1 Lake diversity and fish abundance

A total of 1865 specimens were collected across the 96 sampling events, from 18 species and six families (Table 4.2). The fish assemblages were dominated by *Dorosoma petenense* in five of the six lakes, while in Lake Petcacab it was dominated by *Astyanax bacalarensis*. The most species-rich family was the Cichlidae, with the firemouth cichlid *Thorichthys meeki* the most common cichlid species in all six lakes (Figure 4.2). *Oreochromis* tilapia was only sampled in three of the six lakes, and with only four individuals captured, and an average catch rate across all lakes of only 0.04 individuals per hour (Figure 4.2).

The most species-rich lake was Caobas with 14 out of 18 species (78%, 445 specimens) reported during this survey, and Sta Teresita reported the lowest number, 8 out of 18 (44%) species (Figure 4.3). The accumulation curves at all locations were approaching asymptotes in all lakes, suggesting that sampling was able to provide a reasonable representation of the species richness that it is possible to capture using day-set gillnets, within each lake (Figure 4.3).

Although lakes varied in fish species richness, total species richness in sampling events was positively associated with the concentration of dissolved oxygen and pH (Table 4.3, model #1; Figure 4.4 a,b). This association between species richness and these variables was consistent among lakes (Table 4.3, model #2; Figure 4.4 a,b). There was also a notable positive association between pH and dissolved oxygen across all data (Pearson's correlation, $r = 0.65$, $p < 0.001$; Figure 4.4c). Total species richness in sampling events was positively associated with the concentration of dissolved oxygen and pH (Table 4.3, model #1; Figure 4.4 a,b). Lakes

also differed in the total abundance of fish sampled, but there was no association between *in-situ* measured variables and total fish abundance (Table 4.3, model #3).

**Table 4.2.** Species of fish encountered during the surveys. All are native species, except the tilapia (*Oreochromis* sp.).

| Species | Family | Code | Common name |
|---|---|---|---|
| *Astyanax bacalarensis* | Characidae | Aba | Bacalar tetra |
| *Atherinella alvarezi* | Atherinopsidae | Aal | Gulf silverside |
| *Belonesox belizanus* | Poecillidae | Bbe | pike topminnow |
| *Cribroheros robertsoni* | Cichlidae | Cro | false firemouth cichlid |
| *Cryptoheros chetumalensis* | Cichlidae | Cch | red fin spilurus cichlid |
| *Dorosoma petenense* | Clupeidae | Dpe | threadfin shad |
| *Gambusia yucatana* | Poecillidae | Gyu | Yucatan gambusia |
| *Hyphessobrycon compressus* | Characidae | Hco | Mayan tetra |
| *Mayaheros urophthalmus* | Cichlidae | Mur | Mayan cichlid |
| *Oreochromis* sp. | Cichlidae | Ore | tilapia |
| *Parachromis friedrichsthalii* | Cichlidae | Pfr | yellowjacket cichlid |
| *Petenia splendida* | Cichlidae | Psp | bay snook |
| *Poecilia kykesis* | Poecillidae | Pky | Péten molly |
| *Poecilia mexicana* | Poecillidae | Pme | shortfin molly |
| *Rhamdia guatemalensis* | Heptapteridae | Rgu | pale catfish |
| *Thorichthys meeki* | Cichlidae | Tme | firemouth cichlid |
| *Trichromis salvini* | Cichlidae | Tsa | yellow belly cichlid |
| *Vieja melanura* | Cichlidae | Vme | redhead cichlid |

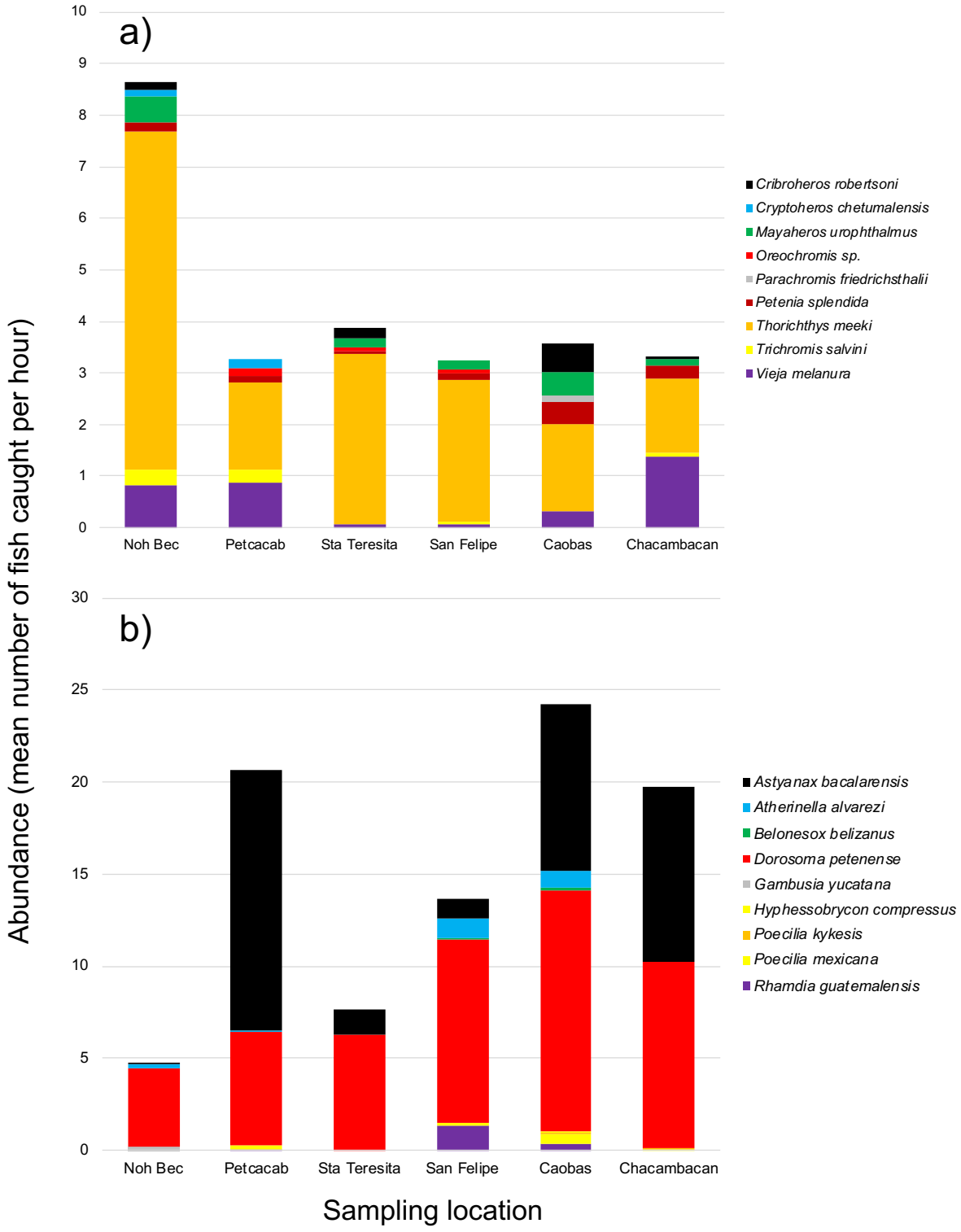


**Figure 4.2.** Summary of the catch composition in each of the lakes, separated into a) cichlids, and b) other species.

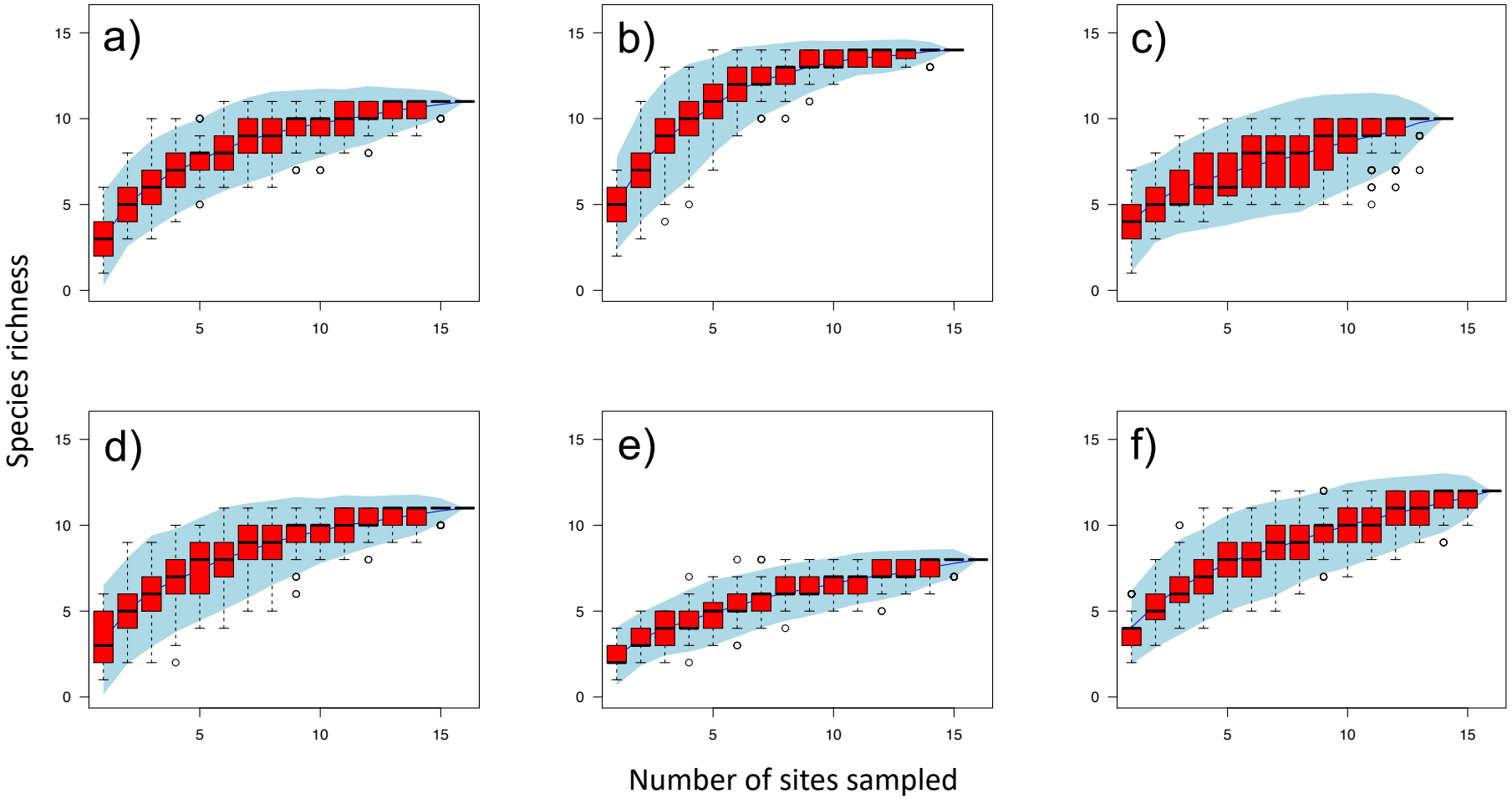


**Figure 4.3.** Species accumulation curves for a) Noh-bec, b) Caobas, c) Chacanbacan, d) Petcacab, e) Sta Teresita, and f) San Felipe.

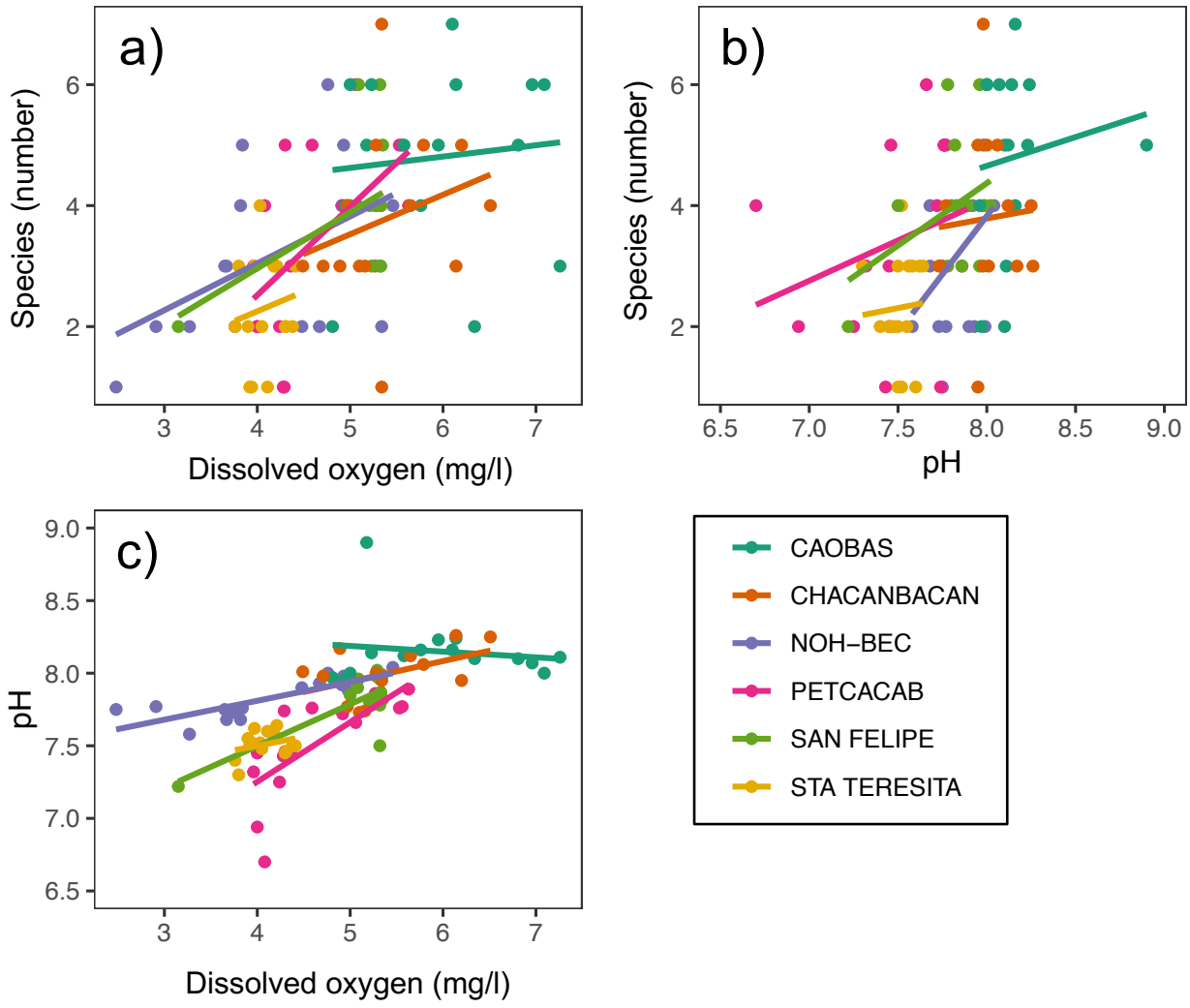


**Figure 4.4.** Associations between a) species richness and dissolved oxygen, b) species richness and pH, and c) dissolved oxygen and pH, across all samples where fish were captured.

**Table 4.3.** Linear models testing association between species number and *in-situ* measured environmental variables, and abundance and *in-situ* measured environmental variables,

| Model# Response | Predictor variables | Df | Sum squares | *F* | *P* |
|---|---|---|---|---|---|
| #1 Species number | Lake | 5 | 54.23 | 6.681 | < 0.001 |
| | pH | 1 | 8.14 | 5.016 | 0.028 |
| | Depth | 1 | 0.10 | 0.062 | 0.804 |
| | Transparency | 1 | 0.25 | 0.151 | 0.699 |
| | Distance to shore | 1 | 0.66 | 0.406 | 0.526 |
| | Dissolved oxygen | 1 | 11.90 | 7.333 | 0.008 |
| | Temperature | 1 | 0.03 | 0.019 | 0.890 |
| | Residuals | 81 | 131.49 | | |
| #2 Species number | Lake | 5 | 54.23 | 6.497 | < 0.001 |
| | pH | 1 | 8.14 | 4.878 | 0.030 |
| | Dissolved oxygen | 1 | 11.76 | 7.046 | 0.010 |
| | Lake x pH | 5 | 0.98 | 0.117 | 0.988 |
| | Lake x Dissolved oxygen | 5 | 6.49 | 0.778 | 0.569 |
| | Residuals | 75 | 125.20 | | |
| # 3 Total abundance | Lake | 5 | 4230 | 3.032 | 0.015 |
| | pH | 1 | 453 | 1.622 | 0.206 |
| | Depth | 1 | 58 | 0.206 | 0.651 |
| | Transparency | 1 | 45 | 0.161 | 0.689 |
| | Distance to shore | 1 | 526 | 1.883 | 0.174 |
| | Dissolved oxygen | 1 | 938 | 3.361 | 0.070 |
| | Temperature | 1 | 262 | 0.94 | 0.335 |
| | Residuals | 81 | 22602 | | |

### *4.3.2 Spatial patterns of community structure among lakes*

Across all the lakes, there were highly significant differences in the community structure ($F_{5,92}$ = 5.47, $r^2$ = 0.24, $P$ < 0.001; Figure 4.5), and in total 10 of 15 pairwise lake differences were significant ($P$ < 0.05) after Bonferroni correction for multiple comparisons (Table 4.4). SIMPER analyses demonstrated that *Dorosoma petenense, Astyanax bacalarensis and Thorichthys meeki* contributed primarily to the differences among lake communities, while invasive *Oreochromis* only contributed < 1% to the differences (Table 4.5).

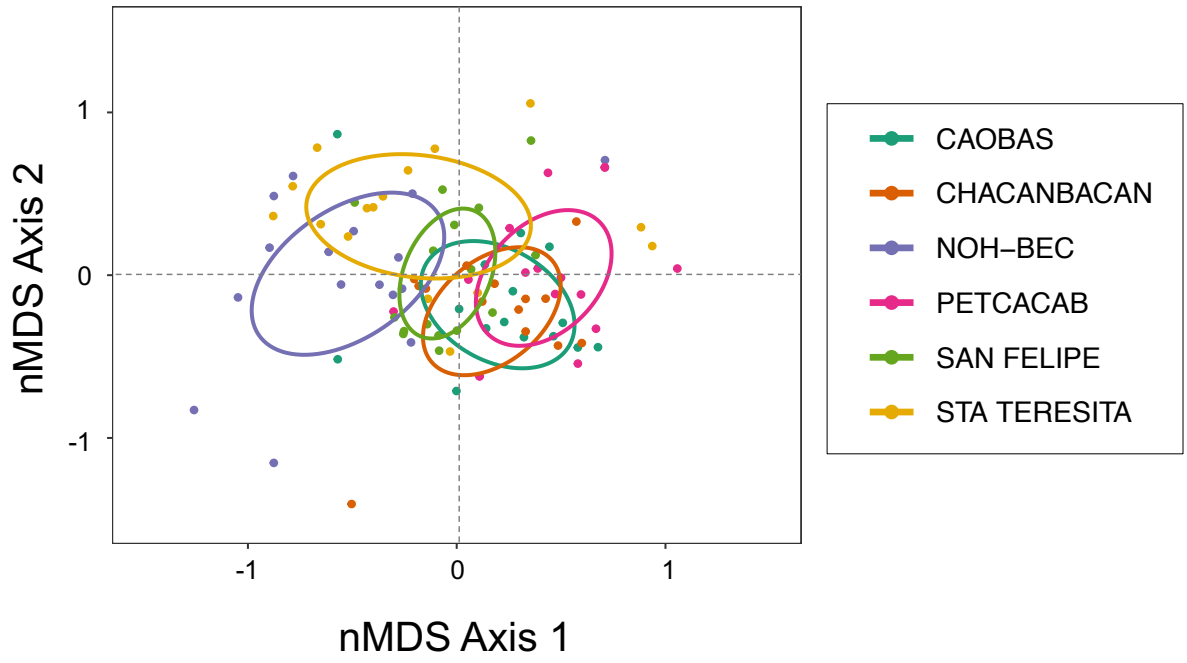


**Figure 4.5.** Non-metric multidimensional scaling ordination plot of fish community structure across the six lakes. Each point represents one sampling event, and closer points indicate more similar species assemblages. Ellipses enclose the standard deviation of the mean. Stress = 0.21.

**Table 4.4.** *Post-hoc* Permanova pairwise tests of difference in fish community structure between lakes. *P* (adjusted) represents *P* values Bonferroni corrected for multiple comparisons.

| Pairwise Comparison | *F* | $r^2$ | *P* | *P* (adjusted) |
|---|---|---|---|---|
| Noh Bec vs Petcacab | 8.227 | 0.215 | 0.001 | 0.015 |
| Noh Bec vs Sta Teresita | 2.460 | 0.076 | 0.035 | 0.525 |
| Noh Bec vs San Felipe | 6.023 | 0.167 | 0.001 | 0.015 |
| Noh Bec vs Caobas | 7.143 | 0.198 | 0.001 | 0.015 |
| Noh Bec vs Chacanbacan | 6.053 | 0.178 | 0.001 | 0.015 |
| Petcacab vs Sta Teresita | 7.831 | 0.207 | 0.001 | 0.015 |
| Petcacab vs San Felipe | 6.295 | 0.173 | 0.001 | 0.015 |
| Petcacab vs Caobas | 2.141 | 0.069 | 0.043 | 0.645 |
| Petcacab vs Chacanbacan | 1.489 | 0.051 | 0.211 | 1.000 |
| Sta Teresita vs San Felipe | 4.990 | 0.143 | 0.001 | 0.015 |
| Sta Teresita vs Caobas | 7.380 | 0.203 | 0.001 | 0.015 |
| Sta Teresita vs Chacanbacan | 8.686 | 0.237 | 0.001 | 0.015 |
| San Felipe vs Caobas | 2.613 | 0.083 | 0.027 | 0.405 |
| San Felipe vs Chacanbacan | 6.889 | 0.197 | 0.001 | 0.015 |
| Caobas vs Chacanbacan | 2.350 | 0.080 | 0.031 | 0.465 |

**Table 4.5.** Average dissimilarity between lakes and % contribution of the total dissimilarity between lakes were calculated using SIMPER.

| Species | Average dissimilarity | % contribution to differences |
|---|---|---|
| *Astyanax bacalarensis* | 2.63 | 4.34 |
| *Atherinella alvarezi* | 12.91 | 21.25 |
| *Belonesox belizanus* | 0.35 | 0.58 |
| *Cribroheros robertsoni* | 0.56 | 0.92 |
| *Cryptoheros chetumalensis* | 1.62 | 2.67 |
| *Dorosoma petenense* | 14.77 | 24.31 |
| *Gambusia yucatana* | 0.36 | 0.59 |
| *Hyphessobrycon compressus* | 0.54 | 0.89 |
| *Mayaheros urophthalmus* | 2.66 | 4.38 |
| *Oreochromis* sp. | 0.58 | 0.95 |
| *Parachromis friedrichsthalii* | 0.22 | 0.37 |
| *Petenia splendida* | 0.26 | 0.42 |
| *Poecilia kykesis* | 1.11 | 1.82 |
| *Poecilia mexicana* | 2.40 | 3.95 |
| *Rhamdia guatemalensis* | 3.05 | 5.02 |
| *Thorichthys meeki* | 10.37 | 17.06 |
| *Trichromis salvini* | 1.26 | 2.07 |
| *Vieja melanura* | 5.10 | 8.39 |

The Canonical Correspondence Analysis revealed strong associations between the composition of the native fish community and environmental variables across lakes (Figure 4.6). In total CCA axis 1 captured 30.9% of the explained variance, while CCA axis 2 captured 25.9%. Positive values on CCA axis 1 were associated with lakes with greater area and less water transparency, while positive values on CCA axis 2 were associated with reduced oxygen and cooler temperatures. In summary models, lake area proved to be more powerful explanatory variable of community structure (greatest variance captured), while substrate, depth and water transparency were also highlighted as important variables and were retained in an optimal model (Table 4.6)

**Table 4.6.** Full and reduced Anova models associated with CCA analyses of all lakes. Note only the variance captured by models is presented.

| | Full model | | | Reduced model | | |
|---|---|---|---|---|---|---|
| Category | Variable | Df | Variance | Variable | Df | Variance |
| *In-situ* | Depth | 1 | 0.036 | Depth | 1 | 0.069 |
| | Transparency | 1 | 0.035 | Transparency | 1 | 0.060 |
| | Distance shore | 1 | 0.042 | Distance shore | - | - |
| | pH | 1 | 0.015 | pH | - | - |
| | Diss. oxygen | 1 | 0.051 | Diss. oxygen | - | - |
| | Temperature | 1 | 0.036 | Temperature | - | - |
| Lake specific | Substrate | 1 | 0.048 | Substrate | 1 | 0.053 |
| | Lake area | 1 | 0.089 | Lake area | 1 | 0.111 |
| Tilapia | Present/Absence | 1 | 0.028 | Present/Absence | - | - |
| | Residual | 83 | 1.840 | Residual | 89 | 2.077 |

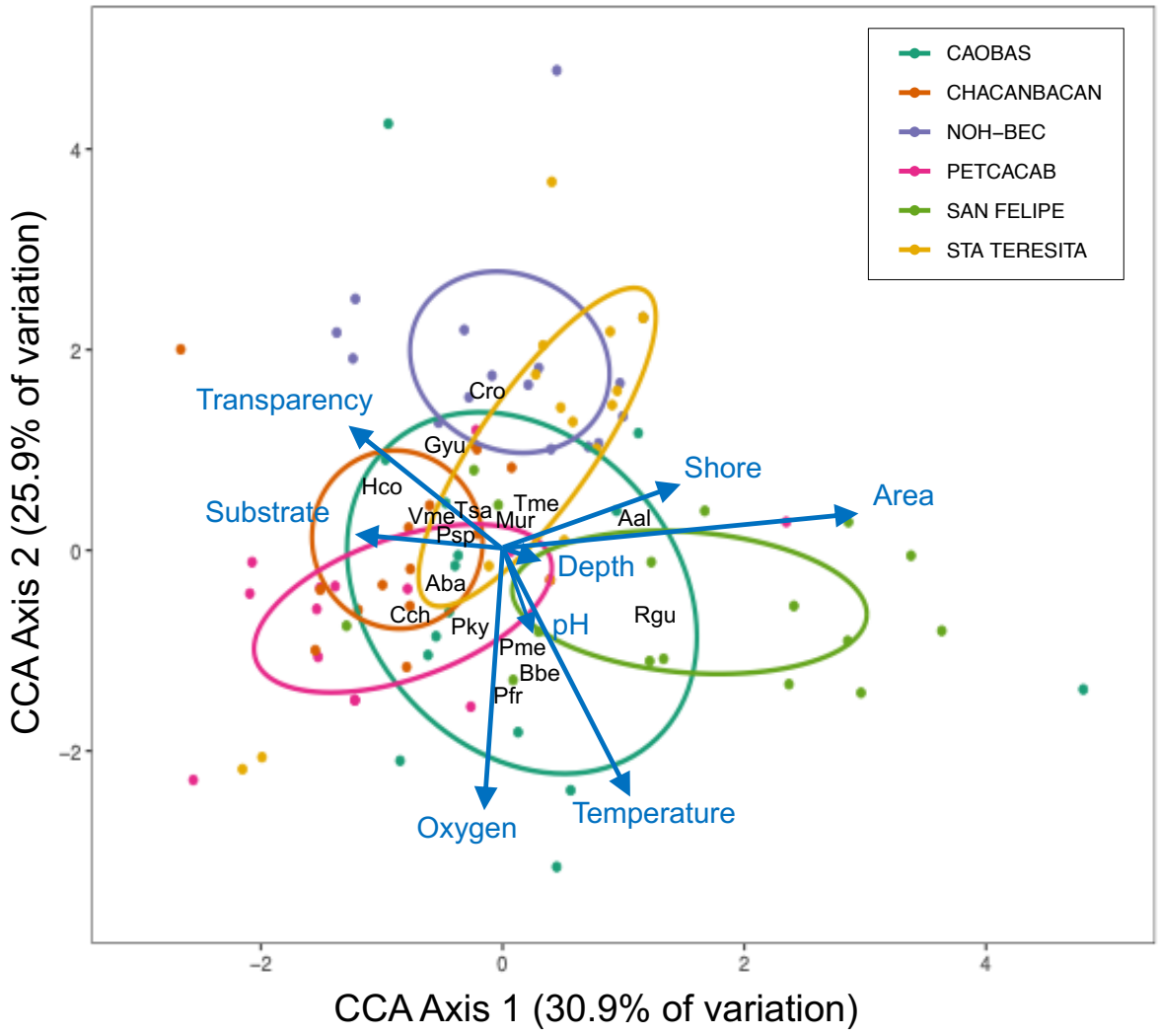


**Figure 4.6.** CCA ordination of the native fish community and environmental variables across the six lakes. a) sampling sites with ellipses representing the standard deviation of the mean of each lake, b) species, and c) all environmental variables. See Table 4.2 for the species codes, and Table 4.1 for environmental variable information.

Variance partitioning analysis provided insight into the relative importance of *in-situ* variables, lake-specific variables, and the presence of *Oreochromis* on the native fish community structure. This demonstrated that variance was explained partially by *in-situ* variables alone (9.7%), partially by lake-specific variables alone (4.7%, namely substrate complexity and lake area), and but Nile tilapia alone was relatively unimportant as an explanatory variable (1.6% alone). There was little covariance between these sets of variables across samples (1.2% total, Figure 4.7), and most of the variance (83%) remained unexplained.

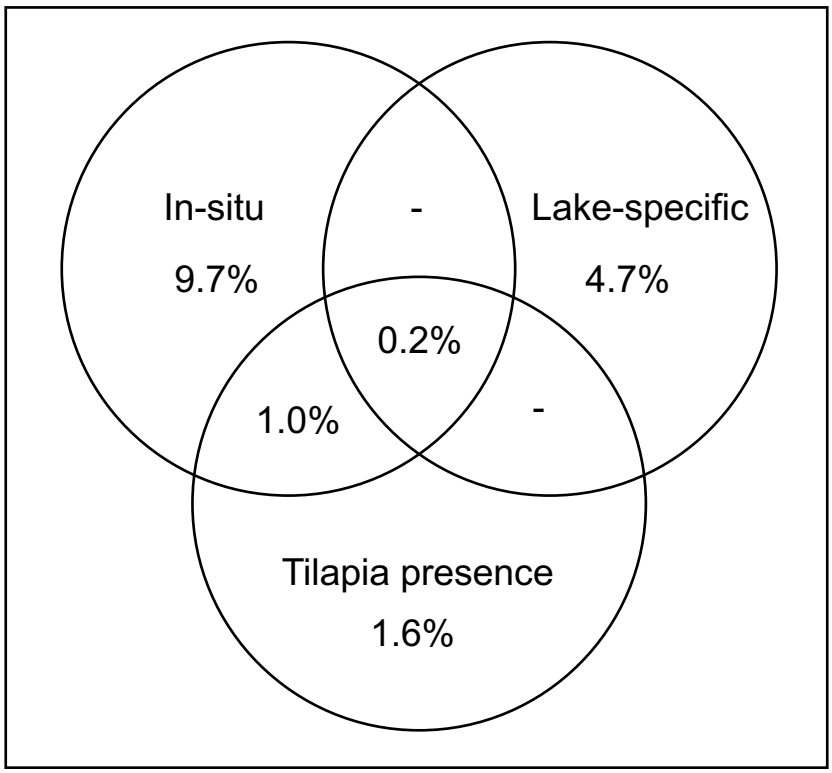


**Figure 4.7.** Variance partitioning analysis of variables explaining community structure across all lakes and sampling events with fish captures. The variables considered were those measured in situ (depth, oxygen, pH, temperature, transparency, distance to shore), those assessed at lake-specific level (area, substrate complexity), and the presence or absence of tilapia.

## 4.4 Discussion

The species assemblages of six lakes in the Yucatan Peninsula were quantified. The species assemblages in each lake were overall similar, being dominated in abundance by open water zooplanktivores including threadfish shad (*Dorosoma petenense*) and Bacalar tetra (*Astyanax bacalarensis*). Additionally, other species that were common to all lakes were the firemouth cichlid (*Thorichthys meeki*), redhead cichlid (*Vieja melanura*) and the bay snook (*Petenia splendida*). There were however differences in the relative abundance of these species, and in the presence or absence of many species between the lakes, and collectively these differences were statistically significant. Variation among the fish communities of the lakes is likely to be a combination of contrasts in the habitats present in each lake, but also differences in the component species from the regional species pool that have had the opportunity to colonise each lake. Sediment cores taken from nearby Lake Chichancanab have revealed the region has been subject to major climate-driven droughts (Hodell *et al.* 2005), which would have affected the integrity of these lake systems. Equally, other large-scale events can affect these habitats such as formation of sink holes due to the underlying karst formations that can lead to instantaneous water loss. One of these events occurred in Lake Chacanbacan in August 2018, approximately one month after sampling (https://bit.ly/3byyPFH). Hence, both differences in the present day habitat, as well as the post-drought dispersal/colonisation processes, are likely to have driven the spatial heterogeneity in fish species composition observed.

### 4.4.1 Environmental correlates of fish diversity within lakes.

Patterns in beta diversity in lakes were significantly dependent on multiple measured variables, including temperature and depth, suggesting fine-scale habitat associations and niche segregation among components of the native fish community (Gamboa-Pérez & Schmitter-Soto 2010). However, overwhelmingly, the key predictor of fish species was oxygen concentration, with higher dissolved oxygen levels corresponding with a greater fish species richness. In principle this could be linked to hypoxic conditions limiting fish movement (e.g. Priede *et al.* 1988). However, since fish can sense dissolved oxygen levels (Wannamaker & Rice 2000), and also exhibit preferences for oxygen-rich habitat (Burleson *et al.* 2001), it is also likely that fish are activity avoiding microhabitats with hypoxic conditions. Also, a positive association between pH and fish species richness was found. Fish distributions are commonly linked to differences in pH, suggesting that there may be pH-dependent habitat preferences (e.g. Warren *et al.* 2010). However, direct experimentation on habitat preferences related to pH would be required to determine if this is taking place in these lakes. Indeed, in these circumstances it is also important to consider the strong positive covariance between dissolved oxygen concentration and pH. Such positive associations between oxygen concentration and pH have been noted in other tropical freshwater lakes (Araoye 2009; Zang *et al.* 2011) and shallow marine environments (Boto & Bunt 1981; Smith *et al.* 2013). They can result from elevated photosynthetic activity of algae increasing oxygen concentration while uptaking $CO_2$, leading to a loss of hydronium ions from the water, thereby raising pH (Smith *et al.* 2013).

### 4.4.2 Low abundance and limits to tilapia invasive success

Perhaps the most notable result from the study is the low capture rate of invasive tilapia across the lakes. In total only four individuals were caught, from Lakes Petcacab, Sta Teresita and San Felipe, although tilapia are also known to have been introduced into Lakes Caobas and Nohbec (Schmitter-Soto & Caro 1997), and environmental DNA has been recovered in relative high densities in Lake Caobas (Chapter 3). It is possible that tilapia are more common in the lakes than the numbers found, but were not represented in catches because of net avoidance behaviour. If so, then alternative sampling methods, such as baited remote underwater video, may have yielded results indicating high densities. Equally, if the nets were set for longer periods then tilapia catches may have increased relative to other species. An alternative possibility is that tilapia were occupying habitats in these lakes that were unsampled, and that greater coverage of the lake environments may identify habitats where these species are abundant. However, since experimental gillnets are routinely used to sample *Oreochromis* species in other regions of the world (e.g. Weyl 1998), and most tilapia tend to be generalist species rather than fine-scale habitat specialists (Shechonge *et al.* 2019), then the data at least provisionally indicate an intrinsically low abundance.

Assuming the low abundance of tilapia in lakes is not a sampling artefact, then the results imply there may be limits to the success of the species in these lakes. In principle, the success of an invasive species may be limited by the availability of suitable trophic resources. Oreochromis tend to feed primarily upon vegetative detritus (Hinojosa-Garro *et al.* 2013), which was abundant in these lakes due to the presence of macrophyte beds. There was also no evidence of these lakes being nutrient poor, with water transparency (~1m) equivalent to the inshore waters of Lake Victoria (Sitoki *et al.* 2010) where Nile tilapia thrive. It is possible

that populations are limited by the predatory species in the lake. The shallow waters of these lakes are occupied by the entirely piscivorous pike topminnow (*Belonesox belizanus*) (Hinojosa-Garro *et al.* 2013), which may represent a predator of juvenile *Oreochromis* that tend to shoal in shallow waters. Larger tilapia are plausible prey of cichlids such as the fully piscivorous bay snook (*Petania splendida*) or the partially piscivorous Mayan cichlid (*Mayaheros urophthalmus*) (Hinojosa-Garro *et al.* 2013). Given evidence that native predators can dramatically limit the invasive success of invasive fish species (Poole & Bajer *et al.* 2019), there is a need for further work to establish the vulnerability of tilapia to these native predators.

### *4.4.3 Concluding remarks*

The apparent low abundance of invasive tilapia in these lakes points towards natural control of the populations, plausibly from natural predators. Therefore, conservation of the indigenous fish communities, and their habitat, may be critical in limiting the spread of invasive species across the region. This study demonstrates, however, the strong link between water quality parameters and fish species richness, therefore implying that changes to the environment could impact on the species assemblages present. Maintenance of the health of these fish communities may require limitations on their use for fisheries, and controls on nitrogen input that can cause eutrophication, leading to reductions in dissolved oxygen concentrations, and ultimately biodiversity loss.

# Chapter 4

# Supplementary information

**Supporting Information 4.1** Abundance of fish species captured during the 96 sampling events across the six lakes. *n* number of individuals sampled across all lakes; numbers of other columns are mean of individuals captured per hour (minimum - maximum).

| Species | *n* | Noh Bec | Petcacab | Sta Teresita | San Felipe | Caobas | Chacambacan |
|---|---|---|---|---|---|---|---|
| *Astyanax bacalarensis* | 562 | 0.06 (0-1) | 14.19 (0-65) | 1.31 (0-12) | 1 (0-5) | 9.06 (0-69) | 9.50 (0-45) |
| *Atherinella alvarezi* | 38 | 0.25 (0-2) | 0.063 (0-1) | - | 1.13 (0-6) | 0.94 (0-10) | - |
| *Belonesox belizanus* | 3 | - | - | - | 0.06 (0-1) | 0.13 (0-1) | - |
| *Cribroheros robertsoni* | 15 | 0.13 (0-1) | - | 0.19 (0-2) | - | 0.56 (0-7) | 0.06 (0-1) |
| *Cryptoheros chetumalensis* | 5 | 0.13 (0-2) | 0.19 (0-2) | - | - | - | - |
| *Dorosoma petenense* | 798 | 4.25 (0-31) | 6.19 (0-13) | 6.31 (0-49) | 9.94 (0-22) | 13.06 (0-39) | 10.13 (0-33) |
| *Gambusia yucatana* | 3 | 0.19 (0-2) | - | - | - | - | - |
| *Hyphessobrycon compressus* | 5 | - | 0.19 (0-2) | - | - | 0.13 (0-1) | - |
| *Mayaheros urophthalmus* | 23 | 0.50 (0-3) | - | 0.19 (0-1) | 0.19 (0-1) | 0.44 (0-2) | 0.13 (0-2) |
| *Oreochromis* sp. | 4 | - | 0.13 (0-1) | 0.06 (0-1) | 0.06 (0-1) | - | - |
| *Parachromis friedrichsthalii* | 2 | - | - | - | - | 0.13 (0-1) | - |
| *Petenia splendida* | 19 | 0.19 (0-1) | 0.13 (0-1) | 0.06 (0-1) | 0.13 (0-2) | 0.44 (0-2) | 0.25 (0-1) |
| *Poecilia kykesis* | 2 | - | - | - | - | 0.06 (0-1) | 0.06 (0-1) |
| *Poecilia mexicana* | 12 | - | - | - | 0.19 (0-2) | 0.50 (0-3) | 0.06 (0-1) |
| *Rhamdia guatemalensis* | 28 | - | 0.06 (0-1) | - | 1.31 (0-4) | 0.38 (0-2) | - |
| *Thorichthys meeki* | 279 | 6.56 (0-26) | 1.69 (0-13) | 3.31 (0-12) | 2.75 (1-6) | 1.69 (0-8) | 1.44 (0-4) |
| *Trichromis salvini* | 11 | 0.31 (0-2) | 0.25 (0-2) | - | 0.06 (0-1) | - | 0.06 (0-1) |
| *Vieja melanura* | 56 | 0.81 (0-3) | 0.88 (0-3) | 0.06 (0-1) | 0.06 (0-1) | 0.31 (0-2) | 1.38 (0-5) |

- Species not sampled.

# Chapter 5

# General Discussion

## 5. 1 General overview

The research presented in this thesis yielded new findings related to invasive *Oreochromis* in freshwater systems of Quintana Roo, Mexico. Since the introduction of tilapia for the purposes of aquaculture and capture fisheries improvement in the 1980s and 1990s, there has been relatively little knowledge about how extensively they have established and their impacts on native fauna. To date, there have been few that has published quantitative estimates of tilapia abundance relative to those of native species (Schmitter-Soto & Caro 1997; Fuselier 2001). Despite the lack of information on the impacts of tilapia on native species, new fish farms continue to establish in the region. This is likely to be leading to new escapes and deliberate introductions to nearby water bodies. It is also possible that rivers in Quintana Roo will be colonised from neighbouring Belize, via the Rio Hondo that separates Belize from Mexico (Esselman *et al.* 2013). The research for this thesis provides useful information regarding the current situation of *Oreochromis* tilapia in the region, by focussing on: (i) competitive interactions between *Oreochromis niloticus* and an indigenous cichlid fish species. (ii) the use and application of eDNA metabarcoding to detect tilapia and quantify diversity of the indigenous freshwater fishes; and (iii) tilapia abundance in six lakes, and how local environmental variables influence total fish diversity and abundance.

## 5.2 Competitive interactions

There have been several studies that have demonstrated competitive dominance of Nile tilapia relative to native fish species (e.g. Martin *et al.* 2010). The research in Chapter 2 demonstrated how Nile tilapia was more aggressive than the Mayan cichlid, consistent with tilapia having a potential negative impact on native species when competiting for limited

resources (McKaye *et al.* 1995; Martin *et al.* 2010). It was notable that activity and behaviour of the Mayan cichlid was strongly negatively influenced by declines in dissolved oxygen decreases, potentiated by temperature increases. By contrast Nile tilapia was able to maintain activity in these conditions. It is possible that this ability to tolerate more extreme environmental conditions has contributed to the biogeographic spread of the species. It is also possible that as temperatures increase by a projected 1°C or more over the coming decades, and anoxic conditions become more widespread, then Nile tilapia may well have an elevated competitive advantage over native species (Rahel & Olden 2008).

Several questions arise from the results. At present, it is unclear if the physiological response of the Mayan cichlid to the environmental conditions is representative of other indigenous species, or if instead they would respond differently. It is also unclear if species would adjust their behaviour if the resources available differed in their value to fish. For example, either of these species may change their behaviour in high predation regimes, or during breeding periods, when shelter may have elevated importance. It is also unclear how differences in body sizes, or density of native cichlids would influence the behaviour of these species. To address these questions would require further experimentation within mesocosms, ideally over longer periods of time where both the behaviour and the fitness consequences of the presence of the other species can be more fully assessed, using fitness proxies such as rates of growth, reproduction and survivorship. It would also be interesting to enrich experimental mesocosms with more indigenous species, therefore resembling invaded and non-invaded aquatic communities. This will permit an experimental evaluation of the value of biodiversity for influencing the success of invasions, thereby offering valuable insights for management and conservation strategies (Preston *et al.* 2012).

### 5.3 Environmental DNA metabarcoding

Within Lake Caobas there was a clear positive association between the number of captures of a species, and the number of eDNA reads. This demonstrates that eDNA metabarcode data can provide a reliable description of the fish assemblage. Additionally, 18 of 20 fish species known from the lake were recovered in the eDNA metabarcoding data, while only 14 of these species were recovered in conventional net sampling. These findings validate results from several eDNA-based surveys that describe the reliability of the technique (Valdez-Moreno *et al.* 2019), and support eDNA-based methods as a low-impact method for surveying freshwater fish communities. The potential for eDNA-based tools will be further enhanced by developments that allow the method to be applied rapidly, and at lower cost than traditional sampling methods (Ardura & Planes 2017; Matter *et al.* 2018).

The work presented in Chapter 3 represents one of the first comparative attempts to use eDNA-metabarcoding to identify the presence of the invasive *Oreochromis* tilapia, while comparing the results with those obtained from the use of traditional survey methods. It was clear from the results that reads assigned to *Oreochromis* were commonplace in the dataset, despite the absence of the species in conventional net captures. Although the absence of tilapia in the net captures may reflect an inability for the gear to reliably sample the tilapia in this environment, the results do confirm the potential for eDNA to reliably describe the presence or absence of invasive tilapia within a lake environment.

More generally, the results demonstrate the potential of environmental DNA to yield of reliable sequences reads in tropical lentic systems subject to relatively high temperatures - where a rapid DNA degradation is expected. The eDNA samples were collected in July, the

month with the highest record of temperature, and daytime water temperatures were typically over 30°C. It is possible that if the survey was performed during months with more moderate temperatures, between November and February, then an eDNA survey may yield an even better representation of the fish assemblages in Lake Caobas.

**5.4 Community structure**

The results reported in Chapters 3 and 4 clearly demonstrate that *Oreochromis* tilapia are widespread in freshwater lakes of the Yucatan Peninsula. It is possible that the distributions reflect active introductions into the lakes for the purpose of developing capture fisheries, but the distributions may reflect dispersal through freshwater or brackish waterways that characterise Quintana Roo. It is now likely that tilapia has extensively colonised most freshwater aquatic systems across the Yucatan peninsula.

However, in the six lakes sampled *Oreochromis* were scarce, with tilapia representing less than 4% of the total number of fish captures, and only being actively caught in three lakes. This is despite the capacity for *Oreochromis* to become abundant in other lakes in the region – historically they comprised 10% or more of the fish biomass of lakes surveyed (Schmitter-Soto & Caro, 1997; Fuselier 2001). Although the low abundance may reflect net avoidance by tilapia in the lakes, it may also indicate that native predator species are regulating the invasive tilapia populations in the surveyed lakes. In support of this hypothesis, it was notable that predator species are more abundant than the Nile tilapia within the fish surveys reported in Chapter 4.

### 5.5. Directions for future research

A key question emerging from this study is the identity of the *Oreochromis* species that are now distributed across the Yucatan Peninsula. Several species of *Oreochromis* are reported to have been introduced into Mexico from Africa, including Nile tilapia (*O. niloticus*), Mozambique tilapia (O. *mossambicus*), blue tilapia (*O. aureus*) and Wami tilapia (*O. urolepis*). For Quintana Roo, reports mention *O. mossambicus* as the first species introduced, followed by *O. niloticus* (Schmitter-Soto & Caro, 1997; Fuselier 2001), but it is possible that any of the *Oreochromis* species in Mexico may have hybridized, and that any of these are now established across aquatic systems of the region. It is of interest to survey the extent of *Oreochromis* genomic diversity across the region and respond to the question of how this diversity is related to the invasive success of the species, and to what extent is possible to identify different *Oreochromis* populations in the region. In case of detection of different populations, then, behavioural trials in experimental mesocosms could be useful to explore the relative competitive ability of genotypic groups with native species across a range of environmental conditions. This future research could be a collaborative initiative between the Tecnologico Superior de Felipe Carrillo Puerto, The Ecosur (Colegio de la Frontera Sur, Quintana Roo) and The University of Bristol, involving local government and Non-Government Organizations.

Quantifying impacts of the tilapia on native species requires long-term experiment in semi-natural ponds, ideally with the composition of native fish communities represented. Experimental ponds with both the presence and absence of the Nile tilapia would be required, alongside acclimatised native predators and generalist species. Underwater video footage could be used to quantify fish interactions, while records are made of the physical and

chemical properties of the water. Several metrics could be used as fitness proxies of tilapia and native species, including survivorship, reproduction and growth. The results would provide explicit evidence of the factors that permit tilapia to thrive, and those that limit population growth. This would enable a test of a key hypothesis that stems from this thesis, that indigenous biodiversity can acts as a barrier to successful establishment of tilapia as a biological invader (*sensu* Kennedy *et al.* 2002).

There is clear potential for eDNA metabarcoding surveys of other freshwater systems of the region, to know more precisely the distribution of tilapia across the region, and to determine how the presence of tilapia associates with the diversity of indigenous species. It will be important to conduct comparative surveys to validate if the eDNA metabarcoding reads counts correlate with the number of captures in other water bodies. This will allow refinement of the technique for surveying this tropical region, enabling more accurate interpretation of results into the future. Authoritative maps of the distribution of *Oreochromis* would allow management and conservation strategies to be drawn up to avoid progressive establishment of these potentially invasive fish. It would be important to sample different lakes in the region during the dry season and during the rainy season when surface waters can become dispersal routes for the invasive tilapia. Sampling using seine nets and traps (such as fyke nets), and complementing the information with eDNA sampling, could offer stronger evidence about the dispersal routes and establishment mechanisms of tilapia in the region.

Although there was an overall positive association between environmental DNA metabarcoding reads of a species in the lake, and the number of captures, the association was relatively weak. Thus, it has not been possible to accurately associate relative read

number with relative biomass, in this study at least. This may be because the amount of DNA released to the environment is different for each species, and depends on body size, movement activity and reproductive rate throughout the year. It may also reflect the ability of primers to bind to template DNA of target species, influencing PCR success in reactions. Clearly there is scope to further refine quantitative approaches that enable eDNA abundance to more accurately reflect actual abundance in aquatic systems, perhaps starting with work in experimental mesocosms.

There are some species with limited natural range in the Yucatan Peninsula, as the *Cyprinodon* species assemblage in Lake Chichankanab that have been drastically perturbed by the presence of tilapia (Schmitter-Soto & Caro 1997). It is also possible that there are other naturally occurring species with limited natural range in the region, which may similarly be under threat from invasive tilapia. To fully understand the threat from tilapia it will be important to identify both sites where *Oreochromis* is already established, as well as the sites that have yet to be colonised by these invaders. Sites without *Oreochromis*, or any other invasive species, and at low risk of being invaded, could be recognized for their ecological integrity as "ark sites". These could be managed as conservation units where human activity is strictly regulated, and conservation activities supported through educational campaigns (Angienda *et al*. 2011, Zengeya *et al*. 2013).

### 5.6 Concluding remarks

It is reasonable to suggest that healthy ecosystems are likely to favour the native fish diversity. In turn this native diversity may be a key factor limiting the expansion of tilapia populations. A high natural fish biodiversity increases the likelihood that native predators are present that

could feed on small-sized tilapia individuals. However, in cases where are warmer conditions and/or low oxygen concentrations, such as those described in the competition experiment, it is possible that tilapia may gain an advantage over native species. The eDNA metabarcoding protocol for native fish assemblages validated for local tropical conditions may complement conventional surveys when mapping invasive species in the region. This may then inform conservation initiatives for native fish assemblages and their aquatic habitats more broadly. This is particularly important for regions where the tilapia farming industry is expanding, and natural disasters like hurricanes and flooding increase the likeability of escapes and accidental releases.

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